Molecular epidemiology of asymptomatic *Plasmodium* species infections in Papua New Guinea

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Dekan

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Summary

In the last two decades, substantial reductions particularly of *P. falciparum* malaria have been achieved world-wide and several countries are now aiming at malaria elimination. A changing malaria epidemiology in elimination settings requires adapting control strategies. A major challenge is the identification of asymptomatic parasite carriers, which constitute a major reservoir sustaining transmission and are often characterized by low parasite densities undetectable by microscopy. Gaps in knowledge of basic epidemiological parameters of asymptomatic and submicroscopic malaria infections in endemic settings, such as the infection rate or the duration of infection, further complicate the planning of control strategies. A second challenge is the control of *P. vivax,* which has proven more resilient to control measures because of its ability to form dormant liver stages. In Papua New Guinea (PNG), *P. vivax* prevalence is among the highest world-wide, but nonetheless *P. falciparum* has been the predominating parasite during the last decades. In recent years both *P. falciparum* and *P. vivax* have been successfully reduced in PNG and are now equally prevalent.

In a first project, this thesis aimed to define the extent of submicroscopic malaria infections in PNG and Tanzania. Half of *P. falciparum* infections in both studies and half of *P. vivax* infections in PNG were not detected by microscopy. Applying two novel ultra-sensitive quantitative PCR (qPCR) assays to samples from a cross-sectional study in Tanzania revealed that a fifth of *P. falciparum* infections were even not detected using standard PCR. Gametocytes, the parasite stage transmitted from human to mosquito, were present in 40% of samples only detected by ultra-sensitive qPCR in Tanzania. A pilot study suggested the presence of such ultra-low density infections also in PNG. The extent of submicroscopic *P. falciparum* infections is thus substantially underestimated even using standard PCR. These results cast doubt on the usefulness of test-and-treat approaches for malaria elimination, which are inherently limited by the sensitivity of the diagnostic used.

A second project of this thesis comprised a detailed analysis of patterns in *P. falciparum* and *P. vivax* infection and disease in school-aged children from PNG. Genotyping of *P. falciparum* and *P. vivax* infections allowed detecting super-infections also in chronically infected parasite carriers. Measuring the molecular force of infection ($_{mol}$FOI), i.e. the incidence of new infections in the blood, permitted quantifying exposure to *P. falciparum* and *P. vivax* on an individual level. Four out of five *P. vivax* infections were attributable to relapsing hypnozoites, stipulating inclusion of liver-clearing drugs in malaria interventions in PNG. More than 10-fold differences in $_{mol}$FOI between the participating villages demonstrated highly heterogeneous malaria transmission in the study area. Even between children from the same village, individual exposure ranged from none to 18 new clones/year for *P.*

falciparum and varied even more for *P. vivax*. This substantial heterogeneity in malaria transmission between neighboring villages, despite almost universal use of insecticide-treated bednets, presents a major challenge for future malaria control strategies in PNG.

In PNG, the age group of children investigated in this thesis (5-10 years) is characterized by substantial semi-immunity particularly to *P. vivax*. Malaria episodes were therefore generally rare and more frequently due to *P. falciparum* despite a lower incidence of new clones compared to *P. vivax*. Higher exposure to *P. falciparum* reduced the risk of experiencing an episode relative to the number of infections received. However, children living in the highest endemic village still carried the main burden of *P. falciparum* episodes due to the higher total number of infections. A concurrent *P. vivax* infection decreased the risk of a *P. falciparum* episode. Age was associated with increasing protection against a *P. vivax* but not a *P. falciparum* episode, suggesting that heterogeneous transmission may abrogate the classical age trends observed previously for *P. falciparum* in PNG.

The large majority of gametocyte carriers in these children were asymptomatic (79% for *P. falciparum* and 92% for *P. vivax*). Gametocytes of both species occurred less frequently in mixed *P. falciparum*/ *P. vivax* infections compared to single-single species infections. Treatment of asymptomatic *P. falciparum* and *P. vivax* infections is required to target the full gametocyte reservoir in PNG.

Longitudinal tracking of individual parasite clones in two cohort studies in PNG children permitted estimating the duration of *P. falciparum* infections. Infections lasted on average 62 days and 38 days in settings of medium and high *P. falciparum* transmission, respectively. Within each study higher exposure was associated with longer infection duration. Infection duration did not change with age. Durations of natural *P. falciparum* infections in PNG children were much shorter than in similarly aged children from Ghana, which may reflect evolutionary adaptation of the parasite to a setting with differing seasonality.

In conclusion, this thesis contributed basic and translational research for malaria surveillance: (i) Novel ultra-sensitive diagnostic assays for *P. falciparum,* designed for use on finger-prick blood samples, are a valuable tool for malaria surveillance. (ii) Parameters for investigating heterogeneity in malaria transmission were validated to inform surveillance response targeted to the local endemicity setting. (iii) Detailed data on patterns in malaria infection and disease in PNG children, on *P. falciparum* infection dynamics and on the *P. vivax* hynozoite reservoir can guide planning of future malaria interventions in PNG.

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Abbreviations

ACT	artemisinin-based combination therapy
AL	artemether-lumefantrine
AQ	amodiaquine
bp	base pair
CE	capillary electrophoresis
CQ	chloroquine
DNA	deoxyribonucleic acid
DTT	dichloro-diphenyl-trichloroethane
EIR	entomological inoculation rate
ELISA	enzyme-linked immunosorbent assay
G6PD	glucose-6-phosphatase dehydrogenase
glurp	glutamate-rich protein
H_e	expected heterozygosity
HRP2	histidine-rich protein 2
IRS	indoor residual spraying
ITN	insecticide-treated bednet
LAMP	loop mediated isothermal amplification
LLIN	long-lasting insecticide-treated bednet
LM	light microscopy
MOI	multiplicity of infection
$_{(mol)}$FOB	(molecular) force of blood-stage infection
$_{(mol)}$FOI	(molecular) force of infection
msp1(F3)	merozoite surface protein 1 (fragment 3)
msp2	merozoite surface protein 2

NASBA	nucleic acid sequence based amplification
PCR	polymerase chain reaction
pPCR	primary PCR
nPCR	nested PCR
qPCR	quantitative PCR
qRT-PCR	quantitative reverse-transcription PCR
PCR-LDR-FMA	PCR - ligase detection reaction - fluorescent microsphere assay
*Pf*EMP1	*Plasmodium falciparum* erythrocyte membrane protein 1
pfs25/pvs25	*Plasmodium falciparum*/ *Plasmodium vivax* 25 kDa ookinete surface antigen
pLDH	*Plasmodium* lactate dehydrogenase
PNG	Papua New Guinea
PQ	Primaquine
RDT	rapid diagnostic test
(m)RNA	(messenger) ribonucleic acid
SP	sulfadoxine-pyrimethamine
TARE-2	telomere-associated repeat element 2
*var*ATS	*var* gene acidic terminal sequence

Chapter 1:

Introduction

Introduction

1.1 A brief overview of malaria epidemiology

Malaria remains one of the most deadly diseases in developing countries, particularly in sub-Saharan Africa, despite substantial gains in malaria control within the last two decades. In 2015, 3.2 billion people remained at risk of malaria and 214 million new cases and 438'000 deaths were reported (World Health Organization, 2015a). Largely as a result of the massive roll-out of insecticide treated bednets (ITNs) to control the mosquito vector *Anopheles spp.*, malaria incidence fell by 37% since the year 2000 (Bhatt et al., 2015; World Health Organization, 2015a). In the same period, the number of global malaria deaths per year was reduced by 48% (Bhatt et al., 2015; World Health Organization, 2015a). With 67% of malaria deaths reported world-wide, African children under the age of five still carry the main burden of malaria morbidity and mortality (Bhatt et al., 2015; World Health Organization, 2015a); however, malaria is no longer the leading cause of death in children in sub-Saharan Africa (Bhatt et al., 2015; World Health Organization, 2015a). Particularly outside of Africa and in countries moving towards malaria elimination, male adolescents or adults replace young children and pregnant women as most vulnerable groups owing to their high occupational risk of becoming infected (Cotter et al., 2013).

Five species of the genus *Plasmodium* cause human malaria: *P. falciparum*, *P. vivax*, *P. malariae*, *P. ovale* and *P. knowlesi*. In Africa, *P. falciparum* is the strongly predominating parasite and responsible for the bulk of malaria-associated mortality and morbidity (Bhatt et al., 2015; Cameron et al., 2015). *P. vivax* is geographically more widespread (Battle et al., 2015; Gething et al., 2012) and less amenable to standard control measures aimed at *P. falciparum,* and thus considered the ultimately more challenging parasite for elimination (Cotter et al., 2013). Although traditionally regarded as the "benign" *Plasmodium* species, *P. vivax* accounts for at least half of clinical malaria cases outside of Africa (World Health Organization, 2015a) and there is a growing body of evidence that also *P. vivax* can occasionally cause severe morbidity (Anstey et al., 2009; Price et al., 2007). *P. malaria* and, with more limited distribution, *P. ovale* are found mostly in sub-Saharan Africa and the Southwest Pacific, but are infrequently also observed in other parts of Asia and the Americas (Mueller et al., 2007a). *P. malariae* and *P. ovale* rarely cause clinical symptoms and are not reliably detected by microscopy or rapid diagnostic test (RDT), hence their true burden may be underestimated (Mueller et al., 2007a). A specific feature of *P. vivax* and *P. ovale* is their capability to form dormant liver stages, called hypnozoites, which can relapse weeks, months or even years after primary infection and are a major source of infection with these species (Robinson et al., 2015; White, 2011). In 2004, zoonotic *P. knowlesi* emerged as the fifth human malaria species and is capable of causing severe disease,

however, transmission so far remains restricted to the south-east Asian jungle (Millar and Cox-Singh, 2015; Singh et al., 2004).

1.2 Malaria diagnosis

A variety of strategies and tools exist for clinical diagnosis of malaria and for measuring malaria endemicity. In countries currently in the control stage of malaria as well as in malaria-free countries, the focus lies on passive monitoring and managing of clinical malaria cases. However, as more and more countries have successfully reduced the burden of clinically patent malaria and aim at malaria elimination (World Health Organization, 2015a), control strategies will need to shift towards active surveillance-response for detection of asymptomatic parasite carriers (Bousema et al., 2014).

Especially in resource-limited field settings, light microscopy (LM) and malaria RDT are the most commonly used tools for differential diagnosis of febrile illness and for generation of malaria prevalence data within national control programs. LM examination of giemsa-stained thick and thin blood smears allows species differentiation of malaria parasites, and can detect >10 parasites/µl blood in when performed by expert microscopists in European reference laboratories (Bejon et al., 2006). In field laboratories or when performed by less-trained personnel, however, sensitivity of LM is considerably reduced and infections below 200 parasites/µl blood are often undetected (Wongsrichanalai et al., 2007). The development of RDTs in the 1990s, which detect one or more of the *Plasmodium* antigens histidine-rich proteins 2 (HRP2), lactate dehydrogenase (pLDH) or aldolase by lateral-flow immuno-chromatography, has facilitated routine and point-of-care malaria diagnosis also in remote field settings. Stability of RDTs in the field has improved over the last decade and their sensitivity now is comparable to that of field microscopy (Bell et al., 2006; World Health Organization, 2015b), but especially detection of non-*falciparum* species remains problematic at densities below 200 parasites/µl blood (World Health Organization, 2015b). Higher parasite prevalence by RDT over LM in community surveys indicates better performance of RDTs compared to LM (Andrade et al., 2010; Fançony et al., 2013; Mwingira et al., 2014), however, the accuracy of RDT test results can be compromised by several factors such as residual *P. falciparum* HRP2 circulating in the hosts's blood after parasite clearance (Bell et al., 2005), high expression of pLDH in mature *P. falciparum* gametocytes (Mueller et al., 2007b), and deletion of *hrp2* in areas of South America (Gamboa et al., 2010).

Molecular methods based on amplification of parasite DNA or RNA can detect parasite densities around or below 1 parasite/µl blood (reviewed in chapter 2) and can reliably discriminate *Plasmodium* species also in mixed-species infections (Mueller et al., 2009). The wider application of molecular detection methods in epidemiological studies in recent years allowed estimating the extent of the previously undetected submicroscopic malaria infection reservoir (Cheng et al., 2015; Okell et al.,

2009, 2012). Mathematical modeling predicts that in low-transmission settings, submicroscopic *P. falciparum* infections make up 80% of all malaria infections and contribute 40% of malaria transmission (Okell et al., 2012), underlining their relevance for successful control programs. Also for *P. vivax*, submicroscopic infections are particularly prevalent in low-transmission areas and on average 67% of infections are not detected by LM (Cheng et al., 2015).

The majority of molecular methods for malaria diagnosis amplify parasite DNA using polymerase chain reaction (PCR) (e.g. Snounou et al., 1993) or loop-mediated isothermal amplification (LAMP) (Polley et al., 2010). While PCR-based methods such as nested PCR (nPCR) or quantitative PCR (qPCR) surpass LAMP in sensitivity and, in case of qPCR, allow quantification of parasite load in the sample (reviewed in chapter 2), they require a more advanced laboratory infrastructure and are thus less applicable in the field compared to LAMP. Similarly, methods for detection of parasite RNA such as (quantitative) reverse transcription PCR (qRT-PCR) (Wampfler et al., 2013) or nucleic acid sequence based amplification (NASBA) (Schoone et al., 2000) reach highest sensitivities but require stringent laboratory practices to avoid sample degradation and cross-contamination, and thus only have limited application in field settings. Recently, high-volume blood sampling has increased the sensitivity of DNA-based methods to that of RNA-based detection, revealing a much larger submicroscopic infection reservoir than previously anticipated (Imwong et al., 2015a, 2015b).

Detection of anti-malarial antibodies in exposed hosts using enzyme-linked immunosorbent assay (ELISA) are promising for use in post-elimination settings as they can sensitively assess past exposure to malaria (malERA Consultative Group on Diagnoses and Diagnostics, 2011).

1.3 Gametocyte detection

Gametocytes are the sexual parasite stage responsible for human-to-mosquito transmission (Figure 1). When ingested by a mosquito, male and female gametocytes mature and fuse in the mosquito midgut, forming a zygote that develops into an ookinete and subsequently oocyst, which eventually gives rises to thousands of mosquito-to-human infective sporozoites. Detection of gametocytes is mainly used to (i) investigate in epidemiological studies the transmission potential of infections, and (ii) to measure post-treatment gametocyte carriage in clinical trials of anti-malarials to evaluate their direct transmission-reducing effect.

Gametocyte detection by LM is problematic especially for *P. vivax*. This is because of generally low parasite densities in natural *P. vivax* infections and the morphological resemblance of *P. vivax* gametocytes and late trophozoites, leading to misclassification of scarce *P. vivax* gametocytemia (World Health Organization, 2010a). Despite the distinct crescent shape of *P. falciparum* mature gametocytes (World Health Organization, 2010a), LM is limited in detecting low-density gametocytemia also for *P. falciparum*. Molecular methods for detection of *P. vivax* and *P. falciparum*

gametocyte-specific RNA by qRT-PCR or quantitative NASBA (QT-NASBA) are at least 50-fold more sensitive than LM, and their application in field studies has revealed substantial submicroscopic gametocyte carriage for both species (Babiker et al., 1999; Beurskens et al., 2009; Koepfli et al., 2015a; Ouédraogo et al., 2007, 2010; Schneider et al., 2004; Shekalaghe et al., 2007; Wampfler et al., 2013). Of the 250 to 300 transcripts that are specifically expressed at different stages of gametocyte development in *P. falciparum* (Lasonder et al., 2002; Silvestrini et al., 2005; Young et al., 2005), the most commonly targeted transcript for detection of *P. falciparum* gametocytes in field studies is the 25 kDa ookinete surface antigen (*pfs25*) mRNA. *Pfs25* is highly and exclusively expressed in female stage V *P. falciparum* gametocytes (Tao et al., 2014; Young et al., 2005) and its mRNA is relatively stable even when blood is collected on filter paper (Jones et al., 2012; Pritsch et al., 2012). Orthologs of *pfs25* are present in other *Plasmodium spp*. including *P. vivax,* which can be targeted for detection of species-specific gametocytes (Beurskens et al., 2009; Mens et al., 2006; Wampfler et al., 2013).

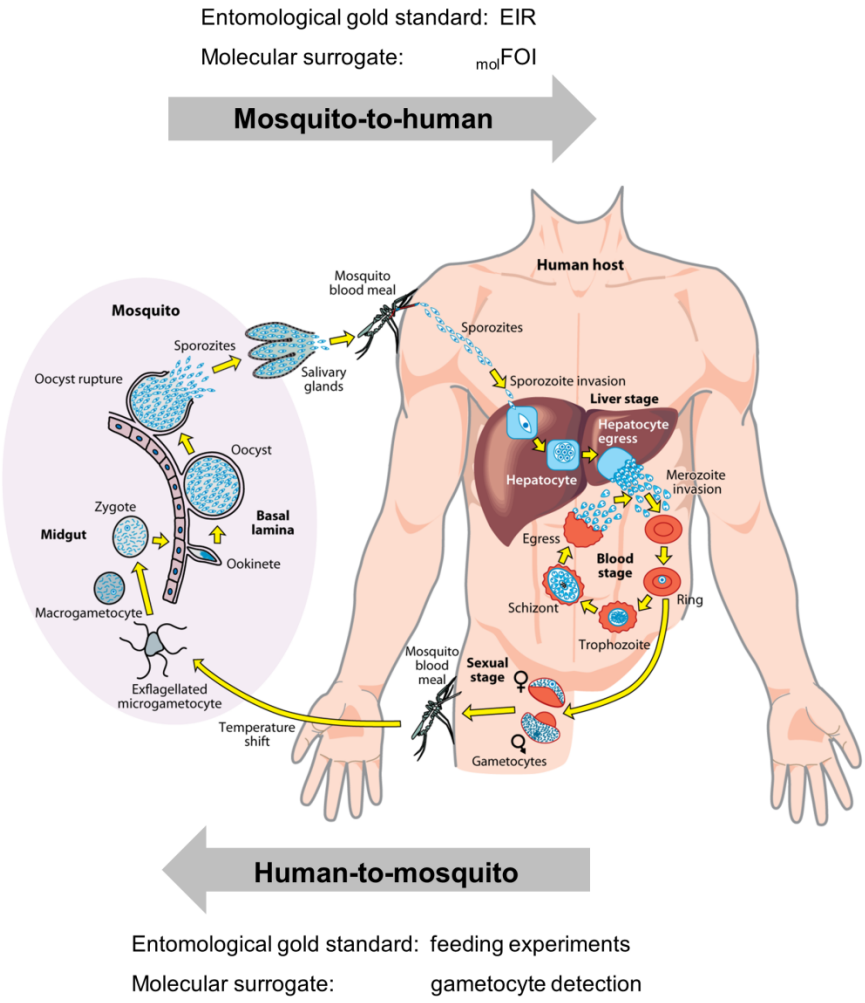

Figure 1. The *Plasmodium* life cycle and measures of transmission. Entomology remains the gold standard, but molecular parameters are easier assessed in epidemiological studies and hence serve as surrogate markers. EIR, entomological inoculation rate; $_{mol}$FOI, molecular force of infection (chapter 1.5). Figure adapted from Boddey and Cowman, 2013.

Application of sensitive molecular methods for gametocyte detection has revealed that submicroscopic *P. falciparum* gametocyte densities are sufficient to infect mosquitoes, albeit at lower rates than LM-detectable gametocyte densities (Bousema et al., 2012; Churcher et al., 2013; Lin Ouédraogo et al., 2016; Ouédraogo et al., 2009; Schneider et al., 2007). Proving the presence of infectious gametocytes undetectable even by molecular methods, a recent study in Burkina Faso showed that 10% of *P. falciparum* infections without any molecularly detectable gametocytes resulted infected mosquitoes (Lin Ouédraogo et al., 2016). Absence of detectable gametocytes thus does not exclude onward transmission of an individual malaria infection to mosquitoes. Nonetheless, investigating factors associated with gametocyte carriage in molecular epidemiologic studies allows identifying high-risk reservoirs of transmission.

1.4 Genotyping of malaria infections

Individual malaria clones can be distinguished in natural infections by genotyping of length-polymorphic markers. Parasite genotyping is widely applied in malaria research, mainly in clinical efficacy trials of new anti-malarials and for molecular monitoring of control interventions. For clinical trials in endemic settings, where people are frequently infected and recurrent parasitemia shortly after treatment is commonly observed, comparing parasite genotypes in pre- and post-treatment blood samples is crucial to distinguish treatment failures from new infections after treatment (Borrmann et al., 2008; World Health Organization, 2008).

For monitoring of malaria control interventions, parasite genotyping allows investigating parasite population structure (Al-Hamidhi et al., 2014; Bakhiet et al., 2015; Delgado-Ratto et al., 2014; Jennison et al., 2015; Koepfli et al., 2015b) as well as determining the multiplicity of infection (MOI), i.e. the number of concurrent clones in a blood sample. MOI has been discussed as a metric for measuring transmission, as the number of co-infecting clones is thought to increase with transmission intensity (Arnot, 1998; Beck et al., 1997; Ntoumi et al., 1995). MOI has also been explored as surrogate marker of anti-malarial immunity because of its age-dependence (Arnot, 1998; Falk et al., 2006; Felger et al., 1999; Mueller et al., 2012; Smith et al., 1999a). Investigating MOI in field studies can therefore potentially help to understand patterns in disease risk. In young children in Tanzania, each additional clone increased the risk for symptomatic malaria episodes (Henning et al., 2004), whereas in older children high MOI seemed to protect from severe malaria and serves as a proxy for exposure (Beck et al., 1997; Genton et al., 2008a; Henning et al., 2004; Manning et al., 2011).

The most commonly used length-polymorphic markers for genotyping of *P. falciparum* are the genes encoding for the merozoite surface proteins 1 and 2 (*msp1* and *msp2*) and the glutamate-rich protein (*glurp*) (Snounou and Beck, 1998; World Health Organization, 2008). Also for *P. vivax*, fragment 3 of the *msp1* gene (*msp1F3*) is one of the most size polymorphic and widely used markers

(Koepfli et al., 2009). Amplification of these markers by nPCR allows highly sensitive discrimination of parasite clones, which differ in the number of intra-genic repeats and hence length of the amplified PCR product. The adoption of capillary electrophoresis (CE) for sizing of PCR products made genotyping of large sample-sets feasible. CE achieves single base pair (bp)-resolution of sizing and allows clear discrimination of allelic families (Falk et al., 2006; Koepfli et al., 2009; Schoepflin et al., 2009).

Marker diversity is measured by calculating the expected heterozygosity (H_e), i.e. the probability that two alleles randomly drawn from a population sample are different (Nei Masatoshi, 1987), according to

$$H_e = \frac{n}{n-1} \cdot \left(1 - \sum p_i^2\right)$$

where n is the number of samples and p_i the frequency of allele i. H_e increases with endemicity along with the number of detected alleles (Schoepflin et al., 2009). In medium- to high-endemic settings, H_e of *Pf-msp2*, *Pf-msp1* and *Pv-msp1F3* ranges between 0.9 and 1 at reasonable sample sizes (Koepfli et al., 2009, 2011a; Schoepflin et al., 2009), and thus chances that two co-infecting clones carry the same allele are usually less than 10%. However, presence of predominant alleles in a population can severely decrease the discriminatory power of genotyping markers especially in low-transmission settings.

1.5 Molecular force of infection ($_{mol}$FOI)

The force of infection (FOI) describes the number of new malaria infections acquired over time, i.e. the infection incidence. FOI is challenging to assess in endemic settings with high *Plasmodium* prevalence, where individuals often carry chronic and asymptomatic infections that complicate detection of super-infections. FOI can be assessed in treatment-to-reinfection cohorts by measuring the time until initially parasite-free participants are re-infected, or by fitting mathematical models to repeat cross-sectional prevalence data (Smith et al., 2010). Recently, high-resolution parasite genotyping in longitudinal cohorts allowed assessing the molecular force of infection ($_{mol}$FOI), i.e. the number of new genetically distinct parasite clones observed in the peripheral blood over time (Koepfli et al., 2013; Mueller et al., 2012). $_{mol}$FOI can be measured in any longitudinal cohort and in spite of pre-existing infections in the host. For *P. falciparum*, $_{mol}$FOI is closely related to the number of infections received from mosquitoes, however, cannot provide information about infections that are controlled before or within the liver. For *P. vivax*, clones observed in the blood stream can originate either from an infective mosquito bite or a relapsing hynozoite from the liver. *P. vivax* $_{mol}$FOI is hence referred to as force of blood-stage infection ($_{mol}$FOB) (Koepfli et al., 2013).

In a cohort study of 1-4 year old children from Papua New Guinea (PNG), *P. falciparum* and *P. vivax* $_{mol}$FOI/$_{mol}$FOB accurately depicted the risk of infection: they exhibited strong seasonality, were dependent on bednet usage and for *P. falciparum* also on age (Koepfli et al., 2013; Mueller et al., 2012). *P. falciparum* $_{mol}$FOI was the strongest predictor of individual risk for a *P. falciparum* episode in these children (Mueller et al., 2012). Higher *P. vivax* $_{mol}$FOB, compared to *P. falciparum* $_{mol}$FOI, illustrated the higher exposure of young PNG children to diverse *P. vivax* over *P. falciparum* parasites despite comparable parasite prevalence (Koepfli et al., 2013).

Due to the high sensitivity of molecular techniques, $_{mol}$FOI may provide a more accurate measure of mosquito-to-human transmission (Figure 1) than the traditional gold standard, the entomological inoculation rate (EIR), especially in settings of low or heterogeneous transmission (Hay et al., 2000; Tusting et al., 2014). Since $_{mol}$FOI can only be determined in longitudinal cohort studies, its wide-spread application in malaria control programs may be limited; however, it is a valuable parameter for validation of more common and easier obtainable parameters such as parasite prevalence.

1.6 Detectability and dynamics of natural malaria infections

Infection dynamics describe the course of malaria infections longitudinally and investigate influencing factors. The two main parameters of malaria infection dynamics are the acquisition rate of new infections (closely related to $_{mol}$FOI/$_{mol}$FOB, chapter 1.5), and the clearance rate of persisting infection, which is inversely correlated to the duration of infection (Felger et al., 2012).

Studying the course of natural malaria infections in endemic settings is complicated by several factors. The majority of malaria infections in endemic areas remain asymptomatic and will go undetected unless communities are actively screened for parasites, since infected hosts will not seek diagnosis and treatment in absence of symptoms. In addition, molecular diagnosis is required for identification of submicroscopic carriers (chapter 1.2). In areas of high transmission and frequent super-infection, monitoring parasite positivity only cannot provide information on the underlying dynamics of clonal infections (Bruce et al., 2000a, 2000b). Super-infecting parasite clones can only be identified using parasite genotyping, and longitudinal tracking of clones is further complicated by their imperfect detectability. Imperfect detectability of clones has been observed in individual blood-samples as well as in longitudinal cohorts, in which clones can be intermittently absent before reappearing in later blood samples (Bretscher et al., 2010; Daubersies et al., 1996; Farnert et al., 1997; Felger et al., 2012; Koepfli et al., 2011b). Imperfect detectability is attributable to periodical sequestration of synchronized *P. falciparum* clones, leading to absence of parasites from the peripheral blood at the time of sampling, or fluctuating parasite densities around the detection threshold of PCR. The detectability of a clone hence largely depends on its density in the host's blood, which in turn is controlled by the immune system of the host (Doolan et al., 2009). Adjusting for imperfect

detectability using mathematical models is therefore crucial for studying the dynamics of natural infections in semi-immune inhabitants of endemic areas (Felger et al., 2012; Sama et al., 2005; Smith and Vounatsou, 2003; Smith et al., 1999b).

Investigating the dynamics of natural malaria infections thus requires longitudinal cohort studies in endemic settings with repeated sampling, the application of technically advanced molecular diagnostics, and sophisticated mathematical analyses. The complexity and cost of such studies likely is one of the reasons why, to this date, some of the most detailed data on the course and duration of malaria infections derives from treatment of neurosyphilis patients by deliberate malaria infection in the pre-antibiotic era ("malaria-therapy"; Collins and Jeffery, 1999a, 1999b; Jeffery and Eyles, 1955). Yet opposed to natural infections in endemic settings, in malaria-therapy the exact time of infection as well as inoculation material is known. The hospitalized patients were easily accessible to researchers and close monitoring of parasitemia was guaranteed. Malaria-therapy patients were usually malaria-naïve and often treated with sub-curative doses of anti-malarial. In addition, no super-infections occurred in this clinical setting, which might alter the course of a pre-existing infection. Therefore, the relevance of malaria-therapy data for natural *P. falciparum* infections in endemic settings is questionable.

1.7 A unique place for malaria research: Papua New Guinea

1.7.1 History of malaria control in PNG

Four of the five human-infecting *Plasmodium* species are found in the highland and lowland areas of Papua New Guinea (PNG), namely *P. falciparum*, *P. vivax*, *P. ovale* and *P. malariae* (Müller et al., 2003). Malaria endemicity varies strongly throughout the country, which is mainly due to differences in temperature depending on altitude. The principal malaria vector in PNG are mosquitoes of the *Anopheles punctulatus* group, but a variety of other species also contribute to transmission and species composition varies across the country (Cooper et al., 2009). In the costal lowlands, transmission is perennial with limited seasonal variation, whereas the highlands are low-endemic and characterized by sporadic outbreaks of malaria (Betuela et al., 2012a; Müller et al., 2003).

Malaria control programs in PNG were initially based on indoor residual spraying (IRS) using dichloro-diphenyl-trichloroethane (DTT) in the 1960s to 1970s, which was eventually combined with mass drug administration using chloroquine (CQ) or amodiaquine (AQ). Cessation of IRS in the 1980s lead to a massive resurgence especially of *P. falciparum*, which replaced *P. vivax* as the most prevalent parasite (Mueller et al., 2005). First CQ-resistant *P. falciparum* and *P. vivax* parasites were observed in 1976 and the late 1980s, respectively, and by the mid 2000s resistance was frequently detected also against combination therapy of CQ or AQ plus sulfadoxine-pyrimethamine (SP)

(Karunajeewa et al., 2008; Marfurt et al., 2007). Along with the release of artemisinin-based combination therapies (ACT), this emergence of drug-resistance prompted a change of first line treatment of uncomplicated malaria in PNG to artemether-lumefantrine (AL), in addition with Primaquine (PQ) in case of uncomplicated *P. vivax* malaria (Papua New Guinea National Department of Health, 2009; World Health Organization, 2010). However, PQ causes hemolysis in individuals with glucose-6-phosphatase dehydrogenase (G6PD)-deficiency (Alving et al., 1956), for which a reliable and affordable point-of-care test is lacking. Futhermore, PQ is not readily available at the majority of health facilities in PNG (Pulford et al., 2013) and thus a large proportion of *P. vivax* malaria in PNG is not adequately treated.

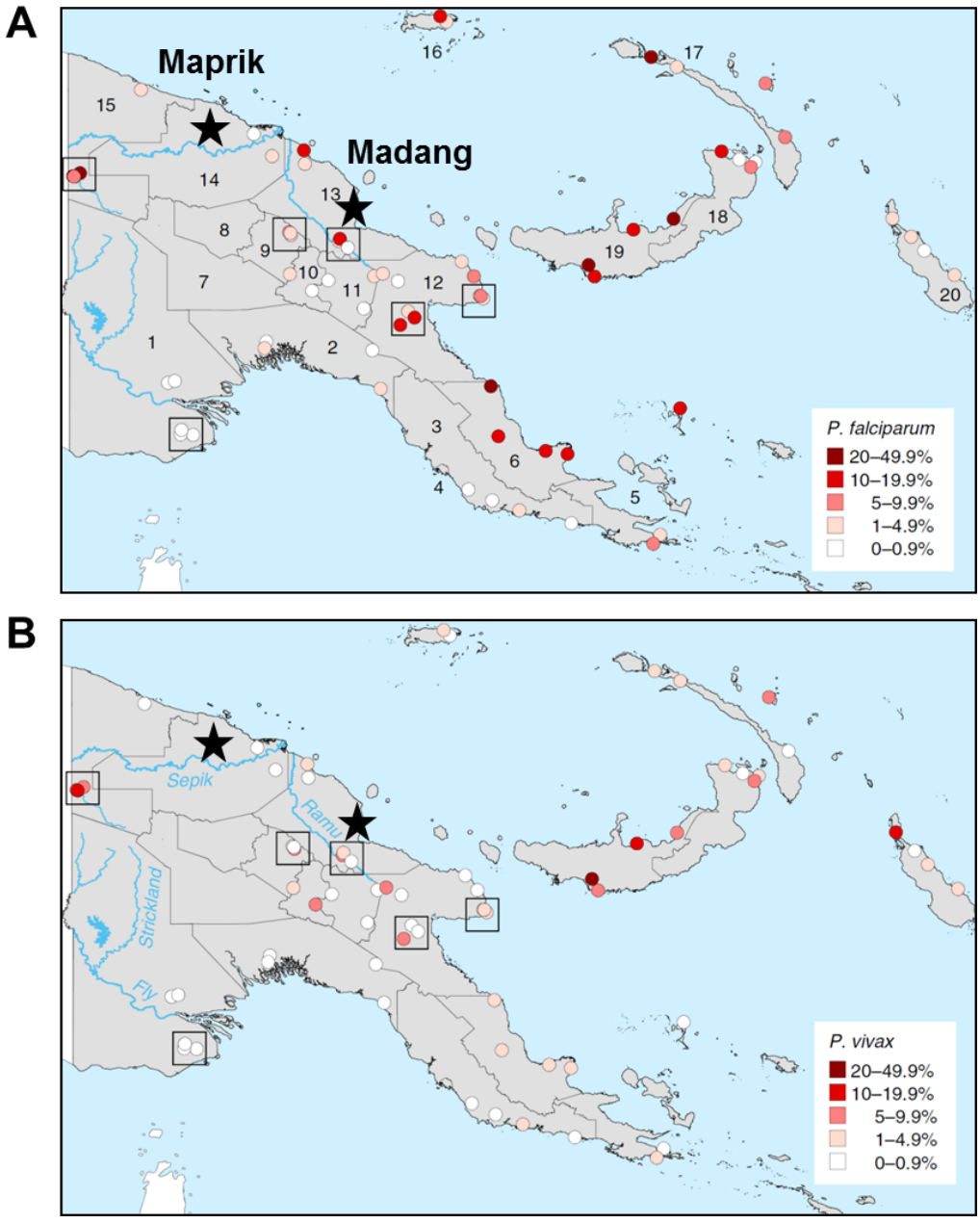

Figure 2. Age-standardized malaria prevalence by LM across PNG in 2008/2009. (A) *P. falciparum* prevalence. (B) *P. vivax* prevalence. Relevant study sites for this thesis are marked with black stars. Figure modified from Hetzel et al., 2015.

In 2004, a grant by the Global Fund to fight AIDS, Tuberculosis and Malaria allowed to re-intensify malaria control efforts in PNG, supporting the countrywide distribution of 2.3 million long-lasting insecticide treated bednets (LLIN) until 2009 (Hetzel et al., 2012). Since 2009, a follow-up Global Fund Round 8 grant supports continued free LLIN distribution (Hetzel et al., 2014), which resulted in 82% LLIN ownership and 48% LLIN usage by 2011 (Hetzel et al., 2014). As result, countrywide prevalence rates by LM dropped to 7% for *P. falciparum*, 4% *P.vivax*, 0.3% *P. malariae* and 0.4% P. ovale (Figure 2), which resembles parasite prevalence in the 1970 after massive IRS (Hetzel et al., 2015). In Madang province, where LM prevalences are comparable to the countrywide mean (7% each *P. falciparum* and *P. vivax*), PCR prevalences in the general population were reduced from 39% in 2006 to 19% in 2010 for *P. falciparum* and 32% to 13% for *P. vivax* (Arnott et al., 2013; Koepfli et al., 2015a; Schultz et al., 2010).

1.7.2 Patterns of malaria infection and disease in PNG

In settings where *P. vivax* and *P. falciparum* are co-endemic, parasite prevalence as well as symptomatic malaria episodes peak earlier in life for *P. vivax* than *P. falciparum* (Karyana et al., 2008; Luxemburger et al., 1996; Maitland et al., 1996; Phimpraphi et al., 2008). Differing mechanisms of anti-malarial immunity development, which remain enigmatic despite decades of intense research, may account for these differences. Also an increased exposure to *P. vivax* blood stage infections due to recurrent relapses, compared to *P. falciparum*, is often given as plausible cause (Koepfli et al., 2013; Robinson et al., 2015). In PNG children, clinical immunity to *P. vivax* is acquired rapidly: uncomplicated disease peaks in children younger than two years and *P.vivax* episodes are rarely reported in children older than five years (Betuela et al., 2012b; Genton et al., 2008b; Lin et al., 2010; Michon et al., 2007). In comparison, the risk of *P. falciparum* episodes increases during early childhood (Lin et al., 2010) and significant reductions are only observed in incidence of high-density infections (>5000 parasites/µl) and symptomatic episodes in young teenagers (Michon et al., 2007). Asymptomatic infections remain common until adulthood for both species in PNG, yet similar to disease patterns, overall *P. vivax* prevalence peaks at younger ages than *P. falciparum* prevalence (Mueller et al., 2009) (Figure 3). Prevalence patterns of *P. malariae* and *P. ovale* resemble those of *P. falciparum* with peak prevalence in older children and young adolescents for both species (Kasehagen et al., 2006; Mueller et al., 2007a).

Because of sympatric transmission of four human *Plasmodium spp*. in PNG, mixed-species infections are common and several studies have tried to address potential effects of species interaction within the human host. However, patterns in the distribution of mixed-species infections in PNG are not clear, with some studies reporting random (Mehlotra et al., 2000, 2002) or non-random (Mueller et al., 2009) occurrence of mixed-species infections. Similarly, it remains unclear how infection with one species alters the risk of infection with another (Bruce et al., 2000c; Smith et al., 2001). A preceding

infection with *P. vivax* seemed to reduce the risk for *P. falciparum* episodes in one study (Smith et al., 2001), but in contrary, PQ treatment and thus lower subsequent *P. vivax* carriage decreased the risk for *P. falciparum* episodes in another (Betuela et al., 2012b). A study in young PNG children described an increased mortality and risk for coma among severe malaria cases in patients carrying mixed *P. falciparum*/*P. vivax* infections compared to single-species infections (Manning et al., 2011). The effects of changing species composition and decreasing prevalence of mixed-species infections alongside successful malaria control programs thus remain difficult to predict.

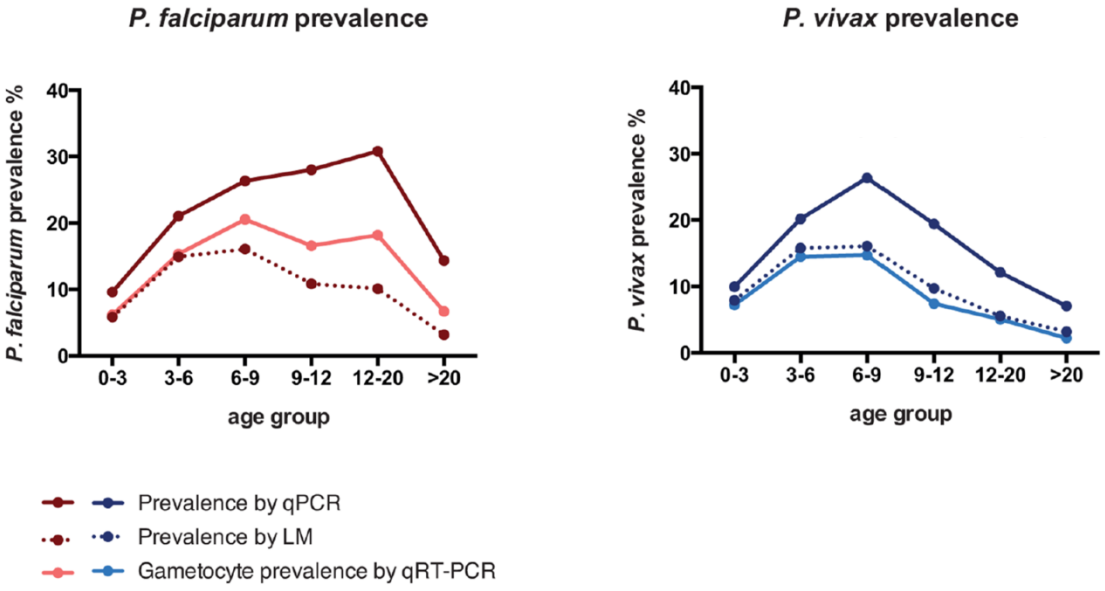

Figure 3. Age-stratified prevalence of *P. falciparum* and *P. vivax* in Madang province, PNG, in 2010. The study took place at the beginning of the (moderate) dry season, roughly two years after LLIN distribution. Figure from Koepfli et al., 2015a.

1.8 The Albinama cohort

Several longitudinal cohort studies have been conducted in the last decade in different geographical settings and age groups in PNG to investigate the molecular epidemiology of *Plasmodium spp*.. This thesis closely revolves around the Albinama cohort, an interventional cohort conducted from 2009 to 2010 in Maprik district, East Sepik province (Figure 2), with the primary aim to quantify the contribution of *P. vivax* relapses to infection and disease in 5-10 year old PNG children (Robinson et al., 2015). Samples from an interventional cohort performed in 2004 in Mugil area, Madang province ("Mugil cohort", Figure 2) (Michon et al., 2007) were analyzed in comparison to the Albinama cohort because of the similar age range of study participants.

The Albinama cohort comprised an initial randomized radical cure treatment over four weeks with CQ and AL plus PQ or placebo (Table 1), followed by 8 months of close active and passive follow-up

(Figure 4) (Robinson et al., 2015). At the beginning of the study, blood-stage parasites were hence cleared in all 504 study participants that completed initial treatment. One half of participants retained *P. vivax* hynozoites in the liver that could relapse during the follow-up period, while in the other half the liver was cleared of hypnozoites by intense PQ treatment. PQ was administered over four instead of two weeks (as recommended by WHO) to assure optimal liver clearance, which had not been achieved after two weeks of PQ treatment an earlier cohort in younger PNG children (Betuela et al., 2012b).

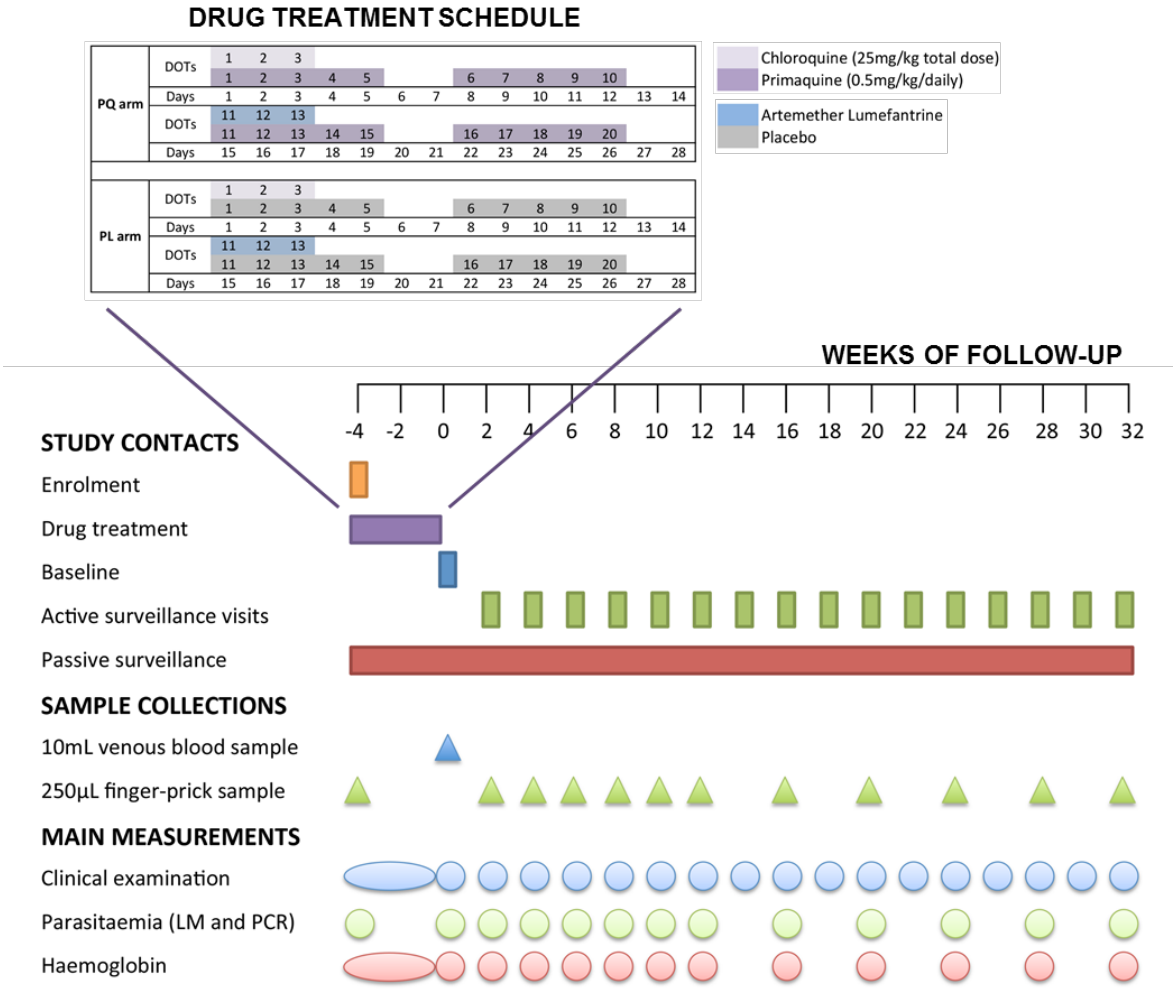

Figure 4. Albinama cohort study design, follow-up schedule and drug administration schedule. All doses of drug treatment were directly observed. Children were actively followed for clinical symptoms in two-weekly intervals throughout the study period, and actively monitored for infection every two weeks in the first 3 months of follow-up and monthy thereafter. Passive surveillance was provided throughout follow-up. PQ arm: Primaquine arm. PL arm: placebo arm. Figure modified from Robinson et al., 2015.

The age group covered by the Albinama cohort (5-10 years) complemented a previous observational cohort conducted in the same study area in younger children (1-4 years) for which detailed molecular epidemiological data exists (Koepfli et al., 2013; Lin et al., 2010; Mueller et al., 2012). The close active as well as passive surveillance of participants in the Albinama cohort, in collaboration with local health centers and health volunteers in all participating villages, allowed registering of all malaria episodes during follow-up together with the type of treatment administered. The Albinama cohort was the first cohort study in PNG that employed specific sampling of blood for molecular detection of gametocytes at all follow-up visits, allowing longitudinal investigation of gametocyte dynamics. The Albinama study thus provides a unique framework for comprehensive, longitudinal and detailed analysis of molecular parasitological parameters, alongside demographic and clinical data for investigation of patterns in malaria infections, transmission and disease.

Table 1. Parasite stage-specific effects of drugs used in the Albinama study.

Drug	Effect on						References
	P. falciparum			*P. vivax*			
	Asexual stages	Gametocytes		Asexual stages	Gametocytes	Hypnozoites	
		immature	mature				
CQ	+	(+)	–	+	+	–	(Bousema and Drakeley, 2011; Pukrittayakamee et al., 2008; Smalley, 1977; White, 2008)
AL	+	+	–	+	+	–	(Bousema and Drakeley, 2011; Eastman and Fidock, 2009; Makanga, 2014; Okell et al., 2008; Pukrittayakamee et al., 2008; Sawa et al., 2013)
PQ	–	–	+	–	+	+	(Graves et al., 2015; Pukrittayakamee et al., 2004, 2008)

–: no effect, +: effective clearance, (+): partially effective clearance

1.9 Aims and objectives of this thesis

The aim of this thesis was two-fold: Firstly, to evaluate and improve the accuracy and precision of molecular methods for detection and genotyping of *P. falciparum* infections for use in epidemiological studies and malaria drug trials. Secondly, to assess patterns in molecular infection parameters of *P. falciparum* and *P. vivax*, as well as infection and transmission dynamics, in semi-immune PNG children to contribute to our understanding of malaria epidemiology and acquisition of anti-malarial immunity in PNG.

The specific objectives of this thesis were as follows:

Objective 1: Development of new ultra-sensitive assays for detection of *P. falciparum* infections

a. To develop DNA-based qPCR assays that circumvent the drawbacks of high-volume sampling and RNA-based detection by targeting multi-copy sequences.
b. To investigate the extent of ultra-low density infections in samples from PNG and in a cross-sectional study in Tanzania.
c. To explore the feasibility of sample pooling using ultra-sensitive detection assays.

Objective 2: Comparative analysis of patterns in *P. falciparum* and *P. vivax* infection and disease in the Albinama cohort

a. To determine molecular epidemiological parameters of malaria infection (prevalence, MOI, $_{mol}$FOI) using *18S rRNA* qPCR, *Pv-msp1F3* and *Pf-msp2* genotyping.
b. To quantify heterogeneity in molecular parameters of infection in the study population.
c. To identify risk factors for malaria infection and symptomatic malaria episodes.
d. To describe the relationship of $_{mol}$FOI and the incidence of malaria episodes.

Objective 3: Modeling the dynamics of natural *P. falciparum* infections in school-aged PNG children

a. To estimate detectability and clearance rates of *P. falciparum msp2*-clones in the Albinama and Mugil cohorts using the triplet model.
b. To quantify the effect of imperfect detectability on estimates of clone acquisition rates.
c. To investigate the relationship of surrogate markers of immunity and exposure, i.e. age and $_{mol}$FOI, with clone acquisition and clearance rates.

Objective 4: Investigating *P. vivax* and *P. falciparum* gametocyte carriage after radical cure

(joint project with Rahel Wampfler, PhD)

a. To quantify gametocyte carriage in submicroscopic and asymptomatic infections using *pfs25* and *pvs25* qRT-PCR in the Albinama cohort.
b. To assess gametocyte production in *P. vivax* relapses and new infections by comparison of treatment arms.
c. To compare gametocyte carriage of *P. falciparum* and *P. vivax* infections and identify risk factors for gametocyte carriage.

Additional project: Validation of the recommendend genotyping procedures for *P. falciparum* recrudescence typing in clinical trials

a. To quantify the extent of amplification bias and minority clone detectability in *P. falciparum* *msp1*, *msp2* and *glurp* PCR.
b. To assess the effect of PCR multiplexing on amplification bias and minority clone detectability.
c. To evaluate the accuracy of sequential typing versus obligate typing of all three markers for recrudescence typing using 44 paired anonymous field samples.

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Chapter 2:

Ultra-sensitive detection of *P. falciparum* infections

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RESEARCH ARTICLE

Ultra-Sensitive Detection of *Plasmodium falciparum* by Amplification of Multi-Copy Subtelomeric Targets

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Abstract

Background

Planning and evaluating malaria control strategies relies on accurate definition of parasite prevalence in the population. A large proportion of asymptomatic parasite infections can only be identified by surveillance with molecular methods, yet these infections also contribute to onward transmission to mosquitoes. The sensitivity of molecular detection by PCR is limited by the abundance of the target sequence in a DNA sample; thus, detection becomes imperfect at low densities. We aimed to increase PCR diagnostic sensitivity by targeting multi-copy genomic sequences for reliable detection of low-density infections, and investigated the impact of these PCR assays on community prevalence data.

Methods and Findings

Two quantitative PCR (qPCR) assays were developed for ultra-sensitive detection of *Plasmodium falciparum*, targeting the high-copy telomere-associated repetitive element 2 (TARE-2, ~250 copies/genome) and the *var* gene acidic terminal sequence (*var*ATS, 59 copies/genome). Our assays reached a limit of detection of 0.03 to 0.15 parasites/μl blood and were 10× more sensitive than standard 18S rRNA qPCR. In a population cross-sectional study in Tanzania, 295/498 samples tested positive using ultra-sensitive assays. Light microscopy missed 169 infections (57%). 18S rRNA qPCR failed to identify 48 infections (16%), of which 40% carried gametocytes detected by *pfs25* quantitative reverse-transcription PCR. To judge the suitability of the TARE-2 and *var*ATS assays for high-throughput screens, their performance was tested on sample pools. Both ultra-sensitive assays correctly detected all pools containing one low-density *P. falciparum*–positive sample, which went undetected by 18S rRNA qPCR, among nine negatives. TARE-2 and *var*ATS qPCRs

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Abbreviations: 18S rRNA, 18S small subunit ribosomal RNA; gDNA, genomic DNA; LAMP, loop-mediated isothermal amplification; LM, light microscopy; LOD, limit of detection; MDA, mass drug administration; MSAT, mass screening and treatment; NASBA, nucleic acid sequence-based amplification; PfEMP1, P. falciparum erythrocyte membrane protein 1; PNG, Papua New Guinea; qPCR, quantitative PCR; qRT-PCR, quantitative reverse-transcription PCR; RDT, rapid diagnostic test; RPA, isothermal recombinase polymerase amplification; TARE-2, telomere-associated repetitive element 2; TZ, Tanzania; varATS, var gene acidic terminal sequence.

improve estimates of prevalence rates, yet other infections might still remain undetected when absent in the limited blood volume sampled.

Conclusions

Measured malaria prevalence in communities is largely determined by the sensitivity of the diagnostic tool used. Even when applying standard molecular diagnostics, prevalence in our study population was underestimated by 8% compared to the new assays. Our findings highlight the need for highly sensitive tools such as TARE-2 and *var*ATS qPCR in community surveillance and for monitoring interventions to better describe malaria epidemiology and inform malaria elimination efforts.

Introduction

Accurate and sensitive detection of malaria parasites is a key factor in planning, targeting, and evaluating malaria control efforts, and requires different strategies at different elimination stages [1–3]. One major challenge is the identification of remaining reservoirs of human-to-mosquito transmission in asymptomatic individuals carrying low-density infections. The true extent of this predominantly submicroscopic reservoir became better defined with the wider application of molecular detection techniques in epidemiological studies [4,5], and its relevance to sustained malaria control has been brought into focus [1–3]. It was recently estimated that submicroscopic but PCR-detectable infections make up 20% of all malaria infections in high-transmission areas and as much as 70% in low-endemic areas, where they contribute 40% of all transmission to mosquitoes [5]. Mass drug administration (MDA) interventions include treatment of these undetected carriers and can thereby reduce parasite prevalence for several months in low- to moderate-prevalence settings, with even longer effects predicted at low transmission levels [6,7]. According to modeling predictions, mass screening and treatment (MSAT) strategies have a lower impact than MDA-based interventions [7], as MSAT is limited by the sensitivity of the diagnostic tool used. A recent study in Burkina Faso found no sustained effect of anti-malarial treatment on incidence of clinical episodes 9 mo after MSAT using conventional diagnosis based on rapid diagnostic test (RDT) [8]. This finding is likely attributable to the large proportion of undetected low-density infections. The true parasite burden could be better defined using nucleic-acid-based diagnostics, but even then, very-low-density infections might be missed. Such low-density infections might be particularly prevalent in areas with a recent and drastic decline in the force of infection of *Plasmodium falciparum*, where high parasite densities and disease are controlled by residual immunity. As more countries successfully reduce malaria prevalence [9], the proportion of low-density infections can be expected to rise, and more sensitive diagnostics that surpass even conventional PCR are urgently needed to detect potential hidden reservoirs.

Of the current molecular detection methods available for malaria diagnosis (summarized in Table 1), RNA-based techniques such as quantitative reverse transcription PCR (qRT-PCR) [10–12], nucleic acid sequence-based amplification (NASBA) [13–15], and ELISA-like hybridization assays [16] reach the highest sensitivities by targeting the highly abundant 18S small subunit ribosomal RNA (18S rRNA). However, because of the unstable nature of RNA, these assays require dedicated and controlled sample collection and storage, and thus have only a limited application in field settings. DNA-based techniques are generally more field-adaptable

Table 1. Assay characteristics and limit of detection (LOD) of published *P. falciparum* detection assays.

Method	Template Molecule	Target Gene	Quantification	LOD (Parasites/µl Blood)	Reference
Nested PCR	DNA	18S rRNA, *dhfr-ts*, 28S rRNA, *stevor*	No	0.1–10	[17–22]
PCR	DNA	mitochondrial DNA	No	0.5	[42]
qPCR	DNA	18S rRNA, *cox1*, *cytb*	Yes	0.02–3	[23–31]
PCR-based	DNA	18S rRNA, *cox1*	Yes/No	0.5–1	[19,37–41]
LAMP[a]	DNA	18S rRNA, mitochondrial DNA	No	1–10	[32–35]
RPA[a]	DNA	18S rRNA	No	4	[36]
qRT-PCR	RNA	18S rRNA	Yes	0.002–0.02	[10–12]
(QT-)NASBA[a]	RNA	18S rRNA	Yes/No	0.02	[13–15]

[a]Isothermal amplification process.
QT-NASBA, quantitative NASBA.

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and include nested PCR [17–22], quantitative PCR (qPCR) [23–31], loop-mediated isothermal amplification (LAMP) [32–35], isothermal recombinase polymerase amplification (RPA) [36], and alternative PCR-based detection methods [19,37–41]. Of the DNA-based assays, only qPCR allows one to robustly quantify copy numbers of the template DNA in the reaction as a measure of parasite load in the sample.

Due to the lower number of target molecules in the sample, DNA-based techniques have a reduced sensitivity compared to their RNA-based counterparts, but sampling for DNA-based diagnosis is more robust. The most prominent molecular marker is the 18S rRNA gene, present at 5–8 copies per genome, depending on the parasite strain [43]. In recent years, several attempts have been made to increase DNA-based PCR sensitivity by sampling larger blood volumes and concentrating the DNA [44], or choosing mitochondrial [19,27,32,42] or nuclear multi-copy PCR targets [40,45]. Already in 1997, Cheng et al. designed a nested PCR that detected the conserved region of the subtelomeric *stevor* gene group, with many copies per genome [46], which had improved sensitivity over single-copy PCRs [47].

We have taken this approach further and have chosen high-copy subtelomeric sequences with the widest possible chromosomal distribution to develop novel qPCR assays for highly sensitive detection and quantification of *P. falciparum* in low-density infections. The telomere-associated repetitive element 2 (TARE-2) is a 1.6-kb-long block consisting of ten to twelve 135-bp repeat units with slightly degenerate sequences, interspersed by two 21-bp sequences [48,49]. The TARE-2 repeat is present at 24 of 28 subtelomeres in the *3D7* culture strain [49], which amounts to approximately 250–280 copies per genome, and is specific to *P. falciparum* strains [48].

The *var* gene family is located primarily in the subtelomere and was chosen to develop a second qPCR with a multi-copy target. The genome of the *3D7* culture strain harbors 59 *var* genes [49], and an estimated 50–150 copies are present in other parasite lines [50,51]. *var* genes encode the *P. falciparum* erythrocyte membrane protein 1 (*Pf*EMP1) and possess a transmembrane domain and one intron, with exons 1 and 2 encoding the extra- and intracellular parts of *Pf*EMP1. In contrast to the highly variable extracellular domain, the intracellular *var* gene acidic terminal sequence (*var*ATS) comprises some well-conserved stretches and can thus be targeted by qPCR [50,51].

With the aim of increasing test sensitivity at least 10-fold and improving the robustness of parasite detection at low densities, we developed two novel qPCR assays using the multi-copy TARE-2 and *var*ATS sequences as targets. We then investigated the potential of both assays to

detect ultra-low-density infections that are beyond the detection limit even of 18S rRNA qPCR. We further hypothesized that the abundance of the PCR target in the parasite genome would counterbalance the diluting effect of sample pooling, and thus tested the suitability of our assays for application to sample pools.

Methods

Ethical Approval

Field samples used for these analyses were derived from a cohort study conducted in Maprik District, Papua New Guinea (PNG), from 17 August 2009 to 20 May 2010 [11] and a cross-sectional survey conducted in Rufiji, Tanzania (TZ), in 2013. Scientific approval and ethical clearance for the PNG cohort study was obtained from the Medical Research and Advisory Committee of the Ministry of Health in PNG (MRAC no. 09.24) and the Ethics Commission of Basel Land and Basel Stadt (no. 237/11). Approval for the TZ cross-sectional study was obtained from the Institutional Review Board of the Ifakara Health Institute, Dar es Salaam, TZ (no. 13-2013). Informed consent was obtained from all study participants in PNG and TZ, for children from parents or legal guardians prior to sampling.

Primer Design and qPCR Conditions

For *var*ATS primer design, all 59 *var*ATS sequences per *P. falciparum* genome (strain *3D7*; PlasmoDB) were aligned using ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/). Within the size-polymorphic *var*ATS domain (size range 1–1.5 kb), the most conserved domain was selected for primer and MGB (minor groove binder) probe design. One wobble each was inserted into the forward primer and probe to improve annealing, whereas the reverse *var*ATS primer matched very well with all *3D7 var*ATS sequences. We expect that only about 40% of *3D7* sequences match sufficiently well with the selected oligonucleotides to yield an amplification product. Attempts to further increase assay sensitivity by using additional wobbles and combinations of primers were not successful. Primer and probe sequences, as well as qPCR mixes and cycling conditions, are listed in S1 Table.

The TARE-2 repeat region was identified in the genome of *P. falciparum* strains *3D7* (National Center for Biotechnology Information) and *IT* (PlasmoDB) using the Tandem Repeats Finder tool (http://tandem.bu.edu/trf/trf.html). TARE-2 sequences of other *P. falciparum* strains were retrieved by BLAST (http://blast.ncbi.nlm.nih.gov) search using *3D7* and *IT* repeat units. All repeat units were aligned using Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/), and primers were designed on the most conserved stretches so that eight nucleotides prior to the 3′ end matched with the majority of repeat sequences. One wobble was inserted into each primer for better annealing. Owing to repeat degeneration and therefore difficult probe design, probe-free SYBR Green–based real-time quantification of amplicons was chosen. Primer sequences and qPCR reaction and cycling conditions are specified in S1 Table. The melt curves of amplicons were inspected in each experiment to detect false positivity. True positive samples differed clearly from primer dimer and unspecific PCR products based on the amplicon's melting temperature (T_m; S1 Fig).

Samples were quantified using a standard curve of plasmid (*var*ATS) or parasite genomic DNA (gDNA; TARE-2). As *var*ATS standard, the *var*ATS amplicon was amplified from *3D7* gDNA and inserted into the TOPO TA vector (Invitrogen). The purified plasmid was diluted to 10^6, 10^4, and 10^2 plasmids/μl in TE buffer. As TARE-2 standard, gDNA of a 10-fold dilution of ring-stage *3D7* parasite culture was used (6.8×10^3 to 6.8×10^{-2} parasites/μl; described in S1 Text).

The reference 18S rRNA qPCR was performed as described previously [11,23], using a MGB probe (6FAM-5′-ACGGGTAGTCATGATTGAGTT-3′-NFQ-MGB) in a total volume of 12 µl. DNA volume matched that of *var*ATS and TARE-2 qPCRs. The amount of target DNA in each sample was calculated from the C_t value using a plasmid standard curve as described above (18S rRNA amplicon inserted in TOPO TA vector [Invitrogen]). *pfs25* qRT-PCR for gametocyte detection was performed as described previously [11].

Analytical Specificity and Sensitivity

The analytical specificity of the TARE-2 and *var*ATS qPCRs was assessed both in silico using BLAST search and experimentally using human gDNA from a healthy, malaria-free volunteer and *P. malariae* and *P. ovale* gDNA (three archived anonymized clinical patient samples each). No amplification from non-falciparum *Plasmodium* or human DNA was observed using the *var*ATS and TARE-2 qPCRs.

For assessment of *P. vivax* cross-reactivity, 14 samples with a low to medium number of genomic *P. vivax* 18S rRNA copies (22–393 *Pv*18S rRNA copies/µl; light microscopy [LM]: 0–219 parasites/µl) were selected from a previously analyzed sample pool [11]. All 14 selected *P. vivax* DNA samples had been diagnosed *P. falciparum*–negative by A18S qRT-PCR. All 14 samples were *var*ATS- and TARE-2-negative.

Analytical sensitivity and qPCR efficiency were validated on dilution rows of (i) in vitro cultured ring stages (*3D7* strain) and (ii) the WHO international standard for *P. falciparum* DNA nucleic acid amplification techniques (*National Institute for Biological Standards and Control*, UK) [27,52]. Details on generation of the dilution rows are presented in S1 Text. TARE-2 and *var*ATS qPCR efficiencies, determined on the *3D7* culture dilution row, were comparable to that of 18S rRNA qPCR; however, all qPCR efficiencies were slightly outside the desirable efficiency range of 90%–105% (Table 2). Efforts to optimize qPCR efficiency by varying primer concentration, annealing temperature, and qPCR volume were not successful.

Field Samples and Nucleic Acid Extraction

In a pilot study, 60 DNA samples from PNG were used for assay validation. They were selected from a larger pool of previously analyzed samples based on their positivity in 18S rRNA qPCR (33 positives, 27 negatives), and we used 18S rRNA copy numbers in these samples to select a wide range of parasite densities [11]. DNA of PNG samples was extracted using the FavorPrep 96-well Genomic DNA Extraction Kit (Favorgen) from blood cell fractions of 50–150 µl, eluted in 200 µl of elution buffer, and stored at −20°C.

The 498 TZ samples were age-stratified randomly selected from the larger cross-sectional sample set, so that each age category contained at least 40 samples. We intended to estimate

Table 2. qPCR details and efficiencies of the 18S rRNA, *var*ATS, and TARE-2 assays.

Assay	Slope	Efficiency	Intercept[a]	R^2	Platform	Amplicon Length[b]	Amplified Copy Numbers in Genome
18S rRNA	−3.63	88.5%	41.09	1.0	TaqMan	221 bp	3
*var*ATS	−3.63	88.6%	34.50	1.0	TaqMan	65 bp	<59[c]
TARE-2	−3.75	84.7%	32.08	0.97	SYBR Green	93 bp	<250–280[c]

[a]Intercept equals the C_t value of the DNA equivalent of five parasites added to the qPCR reaction.

[b]Length of consensus sequence.

[c]Polymorphism in primer binding sites likely does not permit efficient amplification of all genomic copies. Number of target sequences present in parasite genomes from field samples cannot be determined in absence of the respective genome data.

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the overall proportion of *P. falciparum*–positive individuals by each test with a precision given by a CI of ±5%. Samples were collected as 50 μl of whole blood in 250 μl of RNAprotect Cell Reagent (Qiagen) to stabilize RNA. RNA extraction was performed as previously described [11]. DNA was co-extracted during RNA extraction using the RNeasy Plus 96 Kit (Qiagen). DNA was recovered from the gDNA eliminator column after two washing steps according to the QIAamp 96 DNA Blood Kit protocol (500 μl of AW1 buffer, 500 μl of AW2 buffer) and eluted in 100 μl of AE elution buffer.

TARE-2, *var*ATS, and 18S rRNA qPCR were performed once on each TZ DNA sample. If sample positivity did not agree between the three qPCR assays, each qPCR was repeated in duplicate for the discrepant sample, yielding a total of three qPCR replicates for all assays in the discrepant samples. Samples were defined as positive for *var*ATS, TARE-2, or 18S rRNA qPCR if two out of three replicates were positive. For gametocyte detection, *pfs25* qRT-PCR was performed as previously described using 4 μl of RNA [11].

Generation of Pooled Samples

Low-density *P. falciparum*–positive samples (<2 parasites/μl by TARE-2 qPCR, LM negative) were selected from the TZ collection and mixed with four or nine *P. falciparum*–negative blood samples to create pools of five or ten samples. Negative samples were prepared by mixing 50 μl of blood from a malaria-negative blood donor with 250 μl of RNAprotect Cell Reagent (Qiagen) to permit simultaneous DNA and RNA isolation. Per sample, 100 μl of whole blood in RNAprotect Cell Reagent was added to the pool, resulting in a total sample volume of 500 μl or 1 ml (for five- and ten-sample pools, respectively). DNA was extracted from the entire volume of these pools using the RNeasy Plus 96 Kit (Qiagen) as described above, and DNA was eluted in 100 μl (five-sample pools) or 200 μl (ten-sample pools). In total we generated 20 pools of five samples, five of which contained a *P. falciparum*–positive sample, and ten pools of ten samples, two of which contained a positive sample.

Statistical Analyses

Data analysis was performed using R v3.0.2 statistical software. The Mann-Whitney-Wilcoxon test was used to compare for each parasite population (TZ and PNG) the mean T_m of the specific amplicon versus primer dimer. The LOD of qPCR assays, i.e., the concentration at which a sample is detected with 95% confidence, was calculated at using probit analysis of the dilution row results. Proportions of samples positive for the TARE-2, *var*ATS, and 18S rRNA qPCRs in the TZ and PNG datasets were compared using McNemar's Chi^2 test. Correlations of parasite quantity per microliter or template copy number per microliter between assays were calculated using Pearson's product-moment correlation.

Results

Limit of Detection of *var*ATS and TARE-2 qPCRs

Probit analysis was used to determine the LOD, i.e., the concentration at which a sample would be detected with 95% confidence (Fig 1), based on qPCR results from dilution rows of parasite culture and WHO standard material (S3 Table). The *var*ATS and TARE-2 qPCRs were at least 10× more sensitive than standard 18S rRNA qPCR and reached LODs, calculated on the two different dilution rows, of 0.06 and 0.15 parasites/μl (*var*ATS; CI_{95} [0.02–1.07] and [0.05–4.37]) and 0.03 and 0.12 parasites/μl (TARE-2; CI_{95} [not defined] and [0.04–2.06]). Probit analysis of the TARE-2 results using the WHO standard dilution row did not yield a 95% CI because of the steep slope of the regression line. The LOD of 18S rRNA qPCR was calculated at 1.57 parasites/

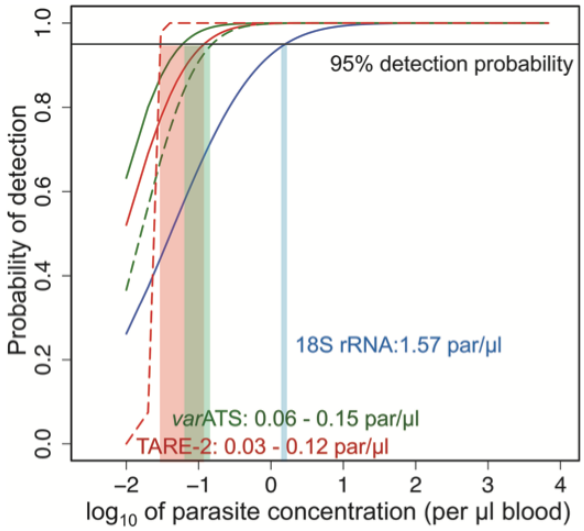

Fig 1. Limit of detection of TARE-2, *var*ATS, and 18S rRNA qPCRs. Dashed lines: based of serial dilution of WHO standard material [52]. Continuous lines: based on serial dilution of ring-stage *3D7* in vitro culture. par, parasites.

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µl (CI_{95} [0.28–626.73]). The TARE-2 and *var*ATS assays can therefore robustly detect as few as 6–24 and 12–30 parasites in 200 µl whole blood, respectively, which is the typical volume normally processed for DNA extraction from fingerprick blood samples without sample concentration.

Detection of Ultra-Low-Density Infections in Maprik District, Papua New Guinea

As pilot study, we compared the ability of the three qPCRs to detect low-density *P. falciparum* infections in 60 DNA samples from PNG. All 33 samples that were positive in 18S rRNA qPCR were also positive using both ultra-sensitive assays. Out of the 27 samples negative by 18S rRNA qPCR, four were positive in *var*ATS qPCR (McNemar's Chi^2, $p = 0.181$). The same four samples plus five additional samples were positive by TARE-2 qPCR, resulting in a significant gain in sample positivity (McNemar's Chi^2, $p = 0.036$). Since samples were not randomly selected but chosen deliberately to include a wide parasite density range, this result does not reflect the true *P. falciparum* prevalence in Maprik District, PNG. Nevertheless the number of additional samples positive for *P. falciparum* demonstrates that a considerable proportion of infections may persist at ultra-low densities and remain undetected by standard qPCR.

Prevalence of Ultra-Low-Density Infections and Gametocyte Carriage in Rufiji, Tanzania

P. falciparum prevalence in Rufiji, TZ, was assessed in 498 samples randomly selected from a larger cross-sectional study conducted in 2013. *P. falciparum* prevalence was higher using ultra-sensitive detection methods as compared to 18S rRNA qPCR, with borderline significance (McNemar's Chi^2, $p_{TARE\text{-}2} = 0.068$, $p_{varATS} = 0.083$). Prevalence values were 25% (CI_{95} [21%–29%]) by LM, 50% (CI_{95} [45%–54%]) by 18S rRNA qPCR, and 58% (CI_{95} [53%–63%])

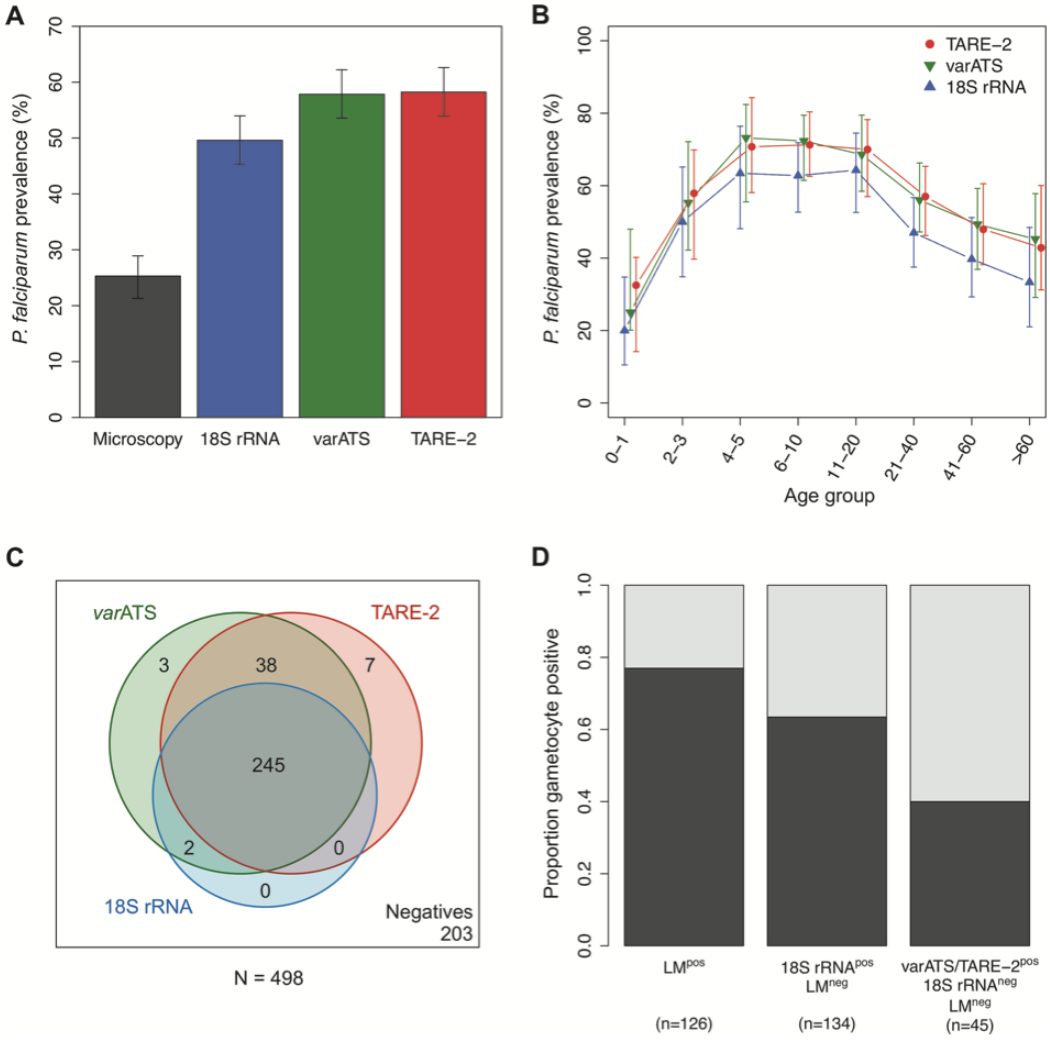

Fig 2. *P. falciparum* prevalence and gametocyte carriage in Rufiji, Tanzania. (A) Overall *P. falciparum* prevalence by different diagnostic methods. Error bars represent 95% CIs. (B) *P. falciparum* prevalence based on TARE-2, *var*ATS, and 18S rRNA qPCRs by age (in years). Error bars represent 95% CIs. (C) Venn diagram of positivity by *var*ATS, TARE-2, and 18S rRNA qPCRs. (D) Proportion of gametocyte carriers by *pfs25* qRT-PCR. Samples were categorized according to the least sensitive method that identified them as *P. falciparum*–positive. In total, 13 of 126 LM-positive samples were not confirmed by any qPCR, and 11 of these also were negative by RDT (SD Bioline Pan pLDH/PfHRP2), thus these samples should be considered false positive by LM. Three samples had to be excluded from the gametocyte analyses because of missing RNA data.

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by *var*ATS qPCR or TARE-2 qPCR (Fig 2A). Applying ultra-sensitive techniques thus revealed a larger submicroscopic infection pool than detected by the routinely used molecular method. Despite a gain in prevalence of 25% over LM, 18S rRNA qPCR still underestimated the true parasite prevalence by 8% without major differences across age groups (Fig 2B). In a total of 295 *P. falciparum* infections, 16% (48 samples) were not detected by 18S rRNA qPCR but only

by *var*ATS or TARE-2 qPCR (Fig 2C). Agreement between assays was very good in the subset positive in 18S rRNA qPCR, with all samples positive in 18S rRNA qPCR detected also by *var*ATS qPCR and all but two by TARE-2 qPCR. The level of agreement between TARE-2 and *var*ATS qPCRs in this sample subgroup was also high, with 79% (38/48) of samples detected by both ultra-sensitive assays.

Quantification of parasite load by *var*ATS and TARE-2 correlated very well with 18S rRNA qPCR quantification in field samples from Rufiji (Pearson's correlation coefficient, $R^2 = 0.98$, CI_{95} [0.97–0.98], and $R^2 = 0.95$, CI_{95} [0.94–0.96], respectively; Fig 3A), as well as with each other ($R^2 = 0.97$, CI_{95} [0.96–0.98]). Correlation of parasite load determined by qPCR and by microscopy was similar for the three assays and ranged from 0.74 (18S rRNA, CI_{95} [0.64–0.81]) to 0.66 (TARE-2, CI_{95} [0.54–0.75]; *var*ATS: 0.68, CI_{95} [0.57–0.80]; Fig 4). Despite the high number of target sequences and slight sequence degeneration, quantification of parasite load is thus feasible using *var*ATS and TARE-2 qPCRs. Parasite loads by TARE-2 qPCR in samples negative by 18S rRNA qPCR were, except for few outliers, within the lowest quartile of all parasite loads by TARE-2 quantification. The same was observed for *var*ATS copy numbers of samples negative by 18S rRNA. When stratified by age, parasite densities or target copy numbers were low in infants up to 1 y, peaked in 2- to 3-y-old children, and decreased thereafter, with the lowest parasite loads observed in the oldest age group (Fig 3B).

The prevalence of gametocytes by *pfs25* qRT-PCR was 40% in all study participants (CI_{95} [36%–45%]). The proportion of *pfs25*-positive samples was highest in samples that were positive by LM, of which 77% (CI_{95} [69%–84%], 97/126) carried gametocytes (Fig 2D). Among submicroscopic infections identified by 18S rRNA qPCR, gametocytes were detected in 63% (CI_{95} [55%–72%], 85/134) of samples. In samples positive only by TARE-2 and/or *var*ATS qPCR, 40% (CI_{95} [26%–56%], 18/45) carried gametocytes. These observations prove that molecularly determined gametocyte carriers are not predominantly found among LM-positive individuals, but rather that an equal number of gametocyte carriers are present in study participants with submicroscopic infections. By use of a routinely used 18S rRNA assay, 16% of asexual infections and 9% of gametocyte carriers would have been missed.

Performance on Sample Pools

To investigate the potential of our assays for a wider application in malaria surveillance or epidemiological field studies, we tested the power of all three qPCR assays to identify *P. falciparum*–positive samples in pools of five or ten samples, each containing one low-density *P. falciparum* infection. 18S rRNA qPCR failed to identify the two positive ten-sample pools and identified only one of five positive five-sample pools. In contrast, *var*ATS and TARE-2 qPCR correctly detected all positive five- and ten-sample pools. No amplification was observed from negative control pools. Our ultra-sensitive assays thus proved suitable for detection of low-grade infections after dilution in nine negative samples. These infections would be missed by 18S rRNA qPCR after pooling. In a setting with 2% *P. falciparum* prevalence, as simulated here, the cost of sample processing and detection can therefore be reduced by at least 70% without loss in sensitivity if ultra-sensitive assays are applied to pools of ten samples.

Discussion

Detecting Low-Density Infections Using Ultra-Sensitive Methods Is Relevant for Malaria Control Efforts

Accurate data on parasite prevalence in the community are imperative for targeting antimalarial interventions and for monitoring their outcome. In this study, we provide first evidence of

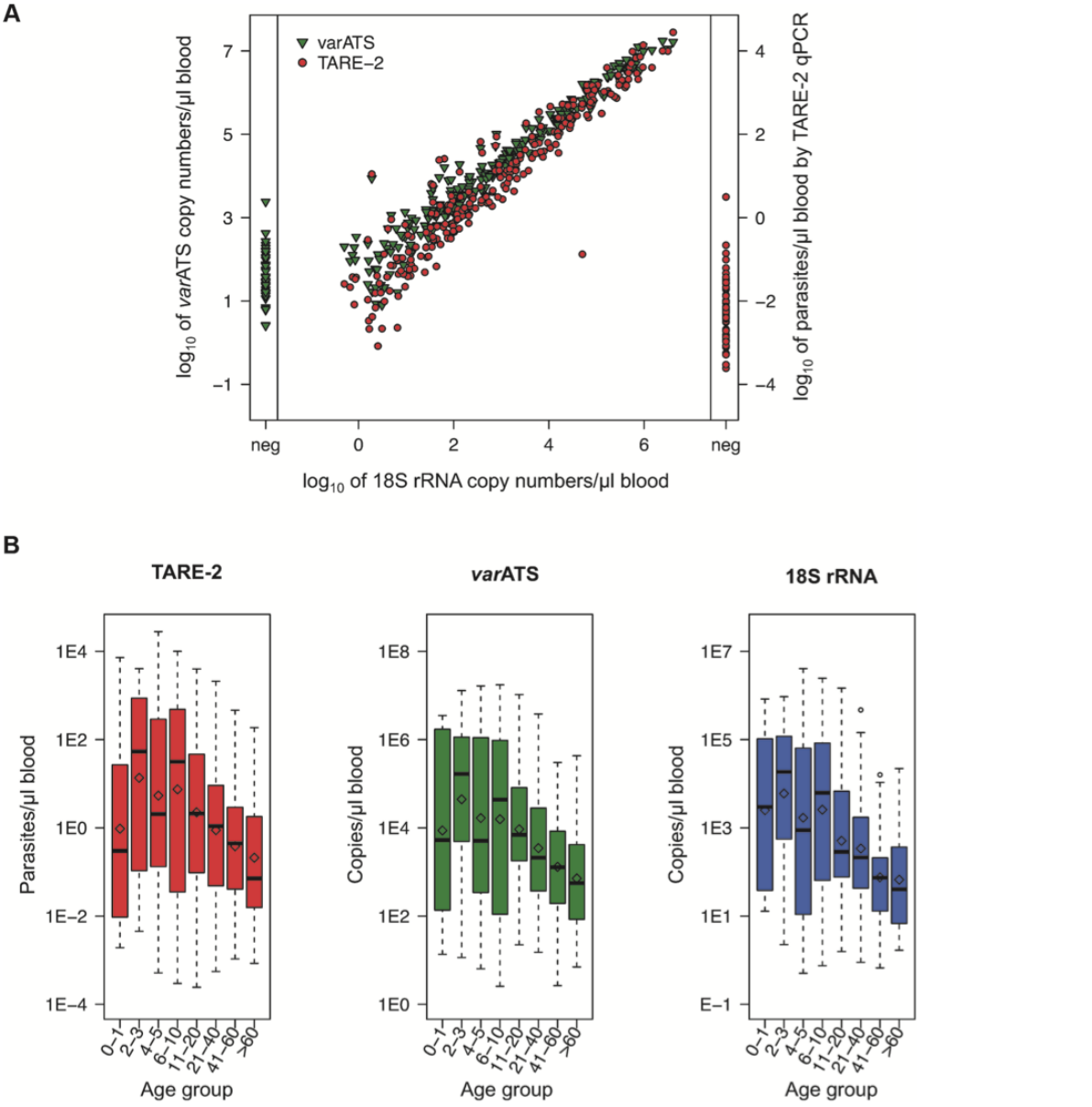

Fig 3. Correlation of parasite quantification using *var*ATS, TARE-2, and 18S rRNA qPCRs and parasite densities in Rufiji, Tanzania. (A) Parasite quantities determined by ultra-sensitive assays and their correlation with 18S rRNA quantification. Quantification was done relative to copy numbers of plasmid standards (18S rRNA, *var*ATS) or a parasite dilution row (TARE-2). Quantities of samples negative in 18S rRNA qPCR but positive in ultra-sensitive assays are shown in the left (*var*ATS) and right (TARE-2) panels. (B) *P. falciparum* densities based on TARE-2, *var*ATS, and 18S rRNA qPCRs by age (in years). The geometric mean in each age group is marked by a diamond; the median is denoted by a black line.

doi:10.1371/journal.pmed.1001788.g003

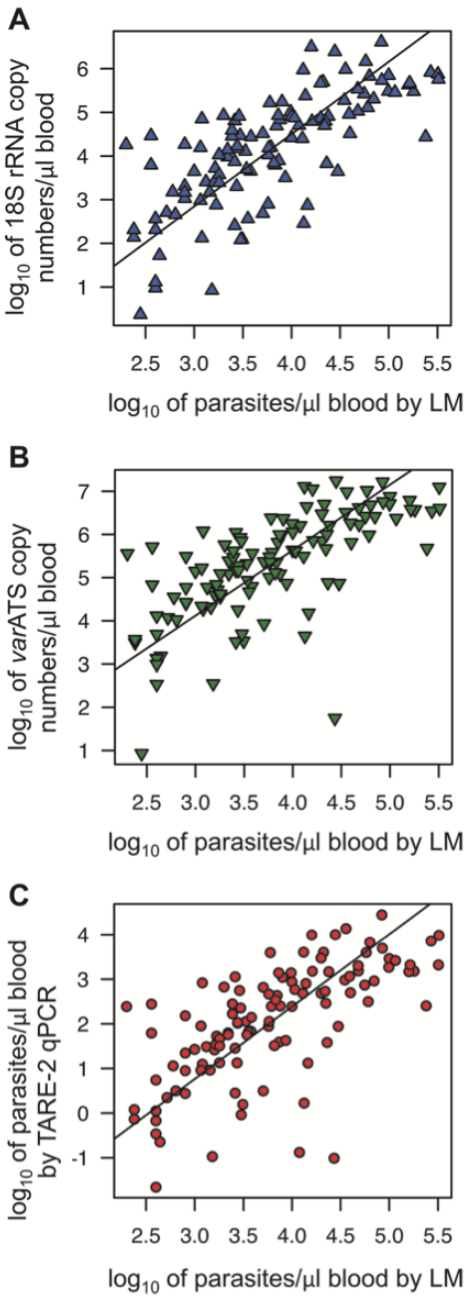

Fig 4. Correlation of parasite quantification by the three qPCR assays and light microscopy. Parasite quantities determined by 18S rRNA (A), *var*ATS (B), and TARE-2 (C) qPCRs and their correlation with parasite density by LM. Quantification by PCR was done relative to copy numbers of plasmid standards (18S rRNA, *var*ATS) or a parasite dilution row (TARE-2). For quantification by LM, 200 fields of a thick film were

examined, and parasite density was calculated assuming 8,000 leucocytes/μl blood. Pearson's product-moment correlation was used to assess correlation strength, and Deming regression was used to calculate regression lines.

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very-low-grade infections in individuals who had previously been considered parasite-free, even after molecular diagnosis, and show that a large proportion of these samples carry gametocytes. In Rufiji, a high-endemic area in TZ, microscopic and submicroscopic infections (by standard 18S rRNA qPCR) each amount to roughly 40% of all *P. falciparum* infections; 16% are of ultra-low density and detected only by TARE-2 and *var*ATS assays. These ultra-low-density infections potentially contribute to transmission, as they represent 9% of the molecularly detected gametocyte carriers. In Maprik District, PNG, 18S rRNA qPCR failed to identify similar quantities of ultra-low-density infections.

A meta-analysis of infection prevalence across the endemicity spectrum has indicated that submicroscopic infections are generally more prevalent in low-transmission settings than in high-transmission areas [5], probably as a result of a recently reduced force of infection and the long duration of asymptomatic untreated infections [53–55]. In such areas, detection of infection, rather than assessment of malaria-associated illness, could serve as a better measure of the malaria burden and a better parameter for surveillance and evaluation [1,2]. Low-density infections can be missed in cross-sectional studies even when using standard 18S rRNA qPCR because parasitemia fluctuates and may occasionally fall below the detection threshold of the assay. Waves of asexual parasitemia and gametocytemia were described in malaria therapy data [56,57]. Accordingly, scanty infections may rise in density at a later time point and increase gametocyte production to detectable levels, leading to higher transmission potential. Improved measures of prevalence using tools for reliable detection of low-density infections can contribute significant information and are important for accurate monitoring and evaluation of malaria control activities, as well as for assessing the potential for onward transmission from human hosts to mosquitoes.

Gametocyte Carriage in Low-Density Infections Emphasizes Their Potential Contribution to Malaria Transmission

Few studies have investigated the transmission potential of submicroscopic infections. Microscopically detectable infections with gametocyte densities below the microscopical threshold can infect mosquitoes, albeit at lower rates than microscopically gametocyte-positive samples (2.3% versus 13.2% infected mosquitoes) [58]. A meta-analysis of mosquito feeding assays conducted in several African countries showed that 27.6% of individuals who lacked microscopically detectable gametocytes were capable of infecting mosquitoes [59]. Similarly, data from the mid-20th century and from two recent studies showed that even blood from infections without any microscopically detectable parasites resulted in 0.2%–3.2% infected mosquitoes [5,60–62]. In a study performed in the Gambia, multiple parasite genotypes were detected in oocysts after feeding mosquitoes on blood seemingly carrying a clonal infection [63]. In that study, multiple gametocyte genotypes were detected in the same blood sample, suggesting that parasite clones undetectable on DNA level produced gametocytes in quantities sufficient to transmit to mosquitoes. Clustering of gametocytes, especially in infections with low gametocyte densities, has been given as a possible explanation for why such infections are able to transmit to mosquitoes [64–66]. A modeling analysis using data from Cameroon found that asexual densities did not predict the proportion of infected mosquitoes, contrary to gametocyte densities, which exhibited a complex and non-linear correlation with transmission success [58,67,68]. Taken together, the available data suggest that all infections should be viewed as

potentially relevant for transmission. The relative contribution of low-density infections to forward transmission to mosquitoes hence may gain substantial importance in areas where these account for a large proportion of infections [5,58]. In our TZ setting, the majority of infections were submicroscopic and harbored 50% of gametocyte-positive samples. TARE-2 and *var*ATS assays identified a so far ignored extent of submicroscopic infective burden, with 40% of these low-key infections carrying gametocytes. We thus argue for including ultra-low-grade infections into the evaluation of malaria interventions and for acknowledging their potential relevance for maintaining transmission, a role that urgently needs experimental clarification.

Sensitivity as a Major Determinant of Prevalence Estimates: Advantages and Limitations of the TARE-2 and *var*ATS qPCRs

Our results highlight the fact that prevalence data are strongly dependent on the sensitivity of the diagnostic technique applied. Even if parasite prevalence is measured using standard qPCR protocols, many low-key infections remain undetected. Standard PCR is widely considered a molecular gold standard of malaria diagnosis complementing LM, the traditional gold standard, yet our results suggest that this notion requires revision. It becomes increasingly clear that the volume of blood analyzed [44] and the use of multi-copy markers to increase the representation of a PCR template in the amplification reaction ([19] and this study) have great influence on the prevalence outcome. Our findings shed new light on MSAT strategies for interruption of transmission in elimination settings, particularly those that rely on RDT-based diagnosis only, as it becomes clear that the ignored proportion of submicroscopic infections is even larger than anticipated. Following a recent MSAT campaign in TZ, RDT-undetected infections were given as a plausible explanation for the short-lived effect on malaria episode incidence [69]. In that study, it was estimated that more than 45% of PCR-detectable infections were missed by RDT, which, given our results, is very likely a substantial underestimation. A major task now consists in adapting molecular methods with enhanced sensitivity to meet the requirements of a robust, field-compatible diagnostic assay. Such tools are becoming increasingly important to determine the infection burden irrespective of endemicity level.

We have presented here two ultra-sensitive qPCR assays for improved detection of low-grade *P. falciparum* infections and their application to sample pools. The *var*ATS qPCR is very robust and highly specific, and allows fast and easy data analysis through the use of a sequence-specific probe. The TARE-2 assay is more susceptible to changes in the chemical composition of the DNA solution and requires melt curve analysis of amplicons, which is a potential drawback, particularly when performed by less-trained personnel. Both assays exhibited slightly suboptimal amplification efficiency despite all optimization efforts, possibly because of a wobble base introduced into primer and probe sequences to improve annealing to the target copies in the genome. Regardless of this inherent low efficiency, sensitivity was superior to that of 18S rRNA qPCR in field samples and on parasite dilution rows. Surprisingly, the TARE-2 qPCR did not outperform the *var*ATS assay despite substantially higher target numbers in the genome. This might be explained by the degenerate sequence of the TARE-2 repeat units or by the clustered distribution of the repeats at chromosome ends. In the *3D7* genome, about ten TARE-2 tandem repeats are present at 24 chromosome ends, and in this arrangement, they may not be separated during DNA extraction. The 59 *var*ATS targets of strain *3D7* also localize to chromosome ends and a few intracellular loci. We assume an equal probability for both targets to be represented in a PCR reaction, but certainly both assays surpass 18S rRNA qPCR, with three copies on different chromosomes targeted by our assay. Because of the need for advanced laboratory infrastructure and staff training, use of our TARE-2 and *var*ATS qPCRs in their current setup is not feasible in remote field settings. However, the assays are ideally suited

for use in reference laboratories, for example for quality assurance or for centralized processing of large sample numbers in sample pools. Several strategies for pooling samples for malaria surveys have been described, comprising one or several pooling steps before [70–72] or after [73,74] DNA extraction. Pooling is severely limited by its inherent diluting effect and is therefore not recommended in the *Malaria* Eradication Research Agenda (malERA) strategy [1]. In low-endemic settings, in particular, where pooling would be most cost- and labor-effective, submicroscopic infections are highly prevalent [5] but are most likely missed in pools because of their low densities. Our *var*ATS and TARE-2 assays proved to be useful for testing pooled samples as they counterbalance the diluting effect through multiple marker copies per parasite. In our hands, even the lowest-density infections diluted with nine negative samples were still detectable. This high sensitivity may be further enhanced by increasing the volume of blood samples and concentrating material before qPCR [44]. The availability of ultra-sensitive assays such as our TARE-2 and *var*ATS qPCRs makes sample pooling without loss in sensitivity feasible and allows achieving higher throughput in the context of limited resources in large-scale field studies. Once similar assays have been developed also for detecting the other human-infecting *Plasmodia*, blood pooling followed by multiplex PCR will further reduce the per-sample cost in studies requiring detection of all four *Plasmodium* species.

Conclusion

In conclusion, we encourage employing assays with enhanced sensitivity, such as the TARE-2 or *var*ATS qPCRs, in any malaria survey aiming to obtain accurate prevalence data and for monitoring intervention success, and recommend them particularly for screening of community samples in areas of low endemicity. The fact that parasites are more prevalent than currently thought has consequences for malaria control efforts, some of which are based on identifying all infected individuals, and this fact must be acknowledged by all users of prevalence data such as health officials, strategy planners, and mathematical modelers. Infections of ultra-low densities in our TZ samples carried gametocytes in 40% of cases, and thus it is highly probable that they can be transmitted to mosquitoes at the time point of the survey or later. Until the infectiousness to mosquitoes of low-density infections has been clarified, applying the most sensitive tools is essential for better defining the true infection burden and informing elimination strategies.

Supporting Information

S1 Fig. Melting temperature of TARE-2 amplicons using DNA samples from two different sources (surveys in Tanzania and Papua New Guinea). Melting temperature (T_m) of true positives (as in positive control/standards) differs significantly from false positive signals (primer dimer, Welch's *t*-test, $p < 0.001$). Owing to the degenerate character of the TARE-2 repeat unit, PCR products vary in sequence composition, which is reflected in slight variations in the T_m of true positives (TZ, 68.6–72.2°C; PNG, 70.0–72.1°C). Different DNA extraction kits and dilution buffers used in the PNG and TZ surveys cause shifts in T_m for both specific amplicons and primer dimer. The mean T_m of true positives and primer dimer was significantly different between the PNG and TZ samples (Welch's *t*-test, $p < 0.001$), while qPCR amplicons amplified from *3D7* DNA standard included on both the TZ and PNG qPCR plates showed no significant differences in their mean T_m. The T_m of specific amplicons and primer dimer was hence established separately for each of our two sets of field samples.
(TIFF)

S1 Table. Oligonucleotide sequences and qPCR conditions for *var*ATS and TARE-2 assays. Primers were purchased from Eurofins. The *var*ATS probe and all qPCR reagents were purchased from Applied Biosystems/Life Technologies.
(DOCX)

S2 Table. STARD checklist for reporting studies of diagnostic accuracy.
(DOCX)

S3 Table. Results of TARE-2, *var*ATS, and 18S rRNA qPCRs on parasite dilution rows.
(DOCX)

S4 Table. Database of light microscopy and qPCR results of the Tanzanian cross-sectional study. Age_Group: in years, corresponding to Figs 2 and 3; 18SrRNA_Quantification: 18S rRNA copy numbers per microliter of blood; *var*ATS_Quantification: *var*ATS copy numbers per microliter of blood; TARE-2_Quantification: parasites per microliter of blood as determined by TARE-2 qPCR; LM_Quantification: parasites per microliter of blood as determined by LM; Pfs25_positivity: 1 indicates positive in *pfs25* qRT-PCR, 0 indicates negative in *pfs25* qRT-PCR.
(XLSX)

S1 Text. Generation of *P. falciparum* dilution rows for determining the limit of detection and qPCR efficiency.
(DOCX)

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Author Contributions

Conceived and designed the experiments: IF. Performed the experiments: NH FM. Analyzed the data: NH IF. Wrote the paper: NH IF. Enrolled patients: FM SS LJR IM. Agree with manuscript results and conclusions: NH FM SS LJR IM IF. ICMJE criteria for authorship read and met: NH FM SS LJR IM IF.

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Editors' Summary

Background

Nearly half the world's population is at risk of malaria, and more than 600,000 people die from this mosquito-borne parasitic infection every year. Most of these deaths are caused by *Plasmodium falciparum*, which is transmitted to people by night-flying *Anopheles* mosquitoes. These insects inject "sporozoites" into people, a parasitic form that replicates inside human liver cells. After a few days, the liver cells release "merozoites," which invade red blood cells, where they replicate rapidly before bursting out and infecting more red blood cells. This increase in parasitic burden causes malaria's characteristic fever, which needs to be treated promptly to prevent anemia and organ damage. Infected red blood cells also release "gametocytes," which infect mosquitoes when they take a blood meal. In the mosquito, the gametocytes multiply and develop into sporozoites, thus completing the parasite's life cycle. Malaria can be prevented by controlling the mosquitoes that spread the parasite and by avoiding mosquito bites. Effective treatment with antimalarial drugs also helps to reduce malaria transmission and is a key component of global efforts to control and eliminate malaria.

Why Was This Study Done?

Planning and evaluating malaria control and elimination efforts relies on having accurate and sensitive methods to measure parasite prevalence—the proportion of a population infected with parasites. It is particularly important to know how many people are carrying low-density infections because although these individuals have no symptoms, they contribute to malaria transmission. In the past, malaria was usually diagnosed by looking for parasites in blood using light microscopy, but molecular tests based on "quantitative polymerase chain reactions" (qPCRs) are now available that detect much lower parasite densities in blood (submicroscopic infections). qPCRs detect parasite-specific DNA sequences in patient blood samples, but reliable detection of low-density infections remains imperfect because the abundance of target sequences in patient samples limits the sensitivity of current qPCR methods. Here, the researchers investigate whether the sensitivity of *P. falciparum* detection using qPCR can be improved by targeting multi-copy genomic sequences—DNA sequences that are repeated many times in the parasite's genetic blueprint.

What Did the Researchers Do and Find?

The researchers developed two new qPCRs for *P. falciparum* by using the telomere-associated repetitive element 2 (TARE-2; 250 copies/genome) and the *var* gene acidic terminal sequence (*var*ATS; 59 copies/genome) as target sequences. Direct comparison of these qPCRs with the standard 18S rRNA qPCR for *P. falciparum*, which targets a gene present at 5–8 copies/genome, indicated that the new assays were ten times more sensitive than the standard assay and could detect as few as 0.03–0.15 parasites/μl blood. Next, the researchers used light microscopy, 18S rRNA qPCR, and the two new qPCRs to look for *P. falciparum* parasites in 498 samples randomly selected from a malaria survey undertaken in Tanzania. Parasite prevalences were 25% by light microscopy, 50% by 18S rRNA qPCR, and 58% by TARE-2 or varATS qPCR. Compared to TARE-2 or *var*ATS qPCR, 18S rRNA qPCR failed to identify 48 infections (16% of infections). Moreover, 40% of the positive samples missed by 18S rRNA qPCR contained gametocytes (detected by a different PCR-

based assay) and therefore came from individuals capable of transmitting malaria parasites to mosquitoes. Finally, to test the suitability of the new ultra-sensitive assays for use in high-throughput screens, the researchers tested performance of the assays on sample pools. Both tests correctly identified all pools containing one low-density *P. falciparum*–positive sample among nine negative samples, whereas 18S rRNA qPCR identified none of these pools.

What Do These Findings Mean?

These findings provide evidence of low-density malaria infections in individuals previously thought to be parasite-free, even after testing with a molecular diagnostic. Notably, in the population considered in this study, the standard 18S rRNA qPCR underestimated parasite prevalence by nearly 10%. The assays developed in this study have some important limitations, however. First, they detect only *P. falciparum*, and malaria control programs ideally need assays that detect all the *Plasmodium* species that cause malaria. Second, because the TARE-2 and *var*ATS qPCRs require advanced laboratory infrastructure, they cannot be used in remote field settings. Nevertheless, because low-density infections are likely to become increasingly common as countries improve malaria control, these findings highlight the need for ultra-sensitive tools such as the TARE-2 and *var*ATS qPCRs for community surveillance and for monitoring the progress of malaria control and elimination programs.

Additional Information

Please access these websites via the online version of this summary at http://dx.doi.org/10.1371/journal.pmed.1001788.

- Information is available from the World Health Organization on malaria (in several languages), including information on malaria diagnosis; the *World Malaria Report 2014* provides details of the current global malaria situation
- The US Centers for Disease Control and Prevention also provides information on all aspects of malaria; its website provides a selection of personal stories about malaria
- Information is available from the Roll Back Malaria Partnership on the global control of malaria and on the Global Malaria Action Plan (in English and French)
- MedlinePlus provides links to additional information on malaria (in English and Spanish)

SUPPLEMENT

S1 Table. Oligonucleotide sequences and qPCR conditions for *var*ATS and TARE-2 assays. Primers were purchased from Eurofins. The *var*ATS probe and all qPCR reagents were purchased from Applied Biosystems/Life Technologies.

	varATS	TARE-2
Oligonucleotide sequences		
Primer-fw (5'-3')	cccatacacaaccaaytgga	ctatgttgcacttacatgcayaat
Primer-rev (5'-3')	ttcgcacatatctctatgtctatct	tgacctaagaagtavaataatgatga
Probe (5'-3')	6-FAM-trttccataaatggt-NFQ-MGB	-
qPCR reaction conditions (final concentration in qPCR mix)		
Total volume	12 (25)[$]	25 (25)[$]
DNA volume	4 (5)[$]	4 (5)[$]
TaqMan® Gene Expression Mastermix	1x	-
Power SYBR® Green mix	-	1x
Primer (each fw & rev)	800 nM	200 nM
Probe	400 nM	-
qPCR cycling conditions		
Pre-incubation	2 min – 50°C	2 min – 50°C
Initial denaturation	10 min – 95°C	10 min – 95°C
Denaturation	15 sec – 95 °C	15 sec – 95 °C
Annealing & Elongation	1 min – 55°C	1 min – 57°C
Number of cycles	45	45
Melt Curve	-	57-95°C, 0.3°C increment
Positivity threshold	0.07	0.07
Standard material for quantification	Plasmid	gDNA of parasite dilution row
Platform	StepOne Plus® Real-Time PCR System (Applied Biosystems)	StepOne Plus® Real-Time PCR System (Applied Biosystems)

[$] Brackets: volumes used for sensitivity and specificity tests on parasite culture and for PNG samples.

The remaining supplementary files can be downloaded from:

http://journals.plos.org/plosmedicine/article?id=10.1371/journal.pmed.1001788#sec028

These include:

S2 Table. STARD checklist for reporting studies of diagnostic accuracy.

S3 Table. Results of TARE-2, *var*ATS, and 18S rRNA qPCRs on parasite dilution rows.

S4 Table. Database of light microscopy and qPCR results of the Tanzanian cross-sectional study.

S1 Text. Generation of *P. falciparum* dilution rows for determining the limit of detection and qPCR efficiency.

Chapter 3:

Micro-geographical heterogeneity in malaria transmission in PNG

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RESEARCH ARTICLE

The complex relationship of exposure to new *Plasmodium* infections and incidence of clinical malaria in Papua New Guinea

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Abstract The molecular force of blood-stage infection ($_{mol}$FOB) is a quantitative surrogate metric for malaria transmission at population level and for exposure at individual level. Relationships between $_{mol}$FOB, parasite prevalence and clinical incidence were assessed in a treatment-to-reinfection cohort, where *P.vivax* (*Pv*) hypnozoites were eliminated in half the children by primaquine (PQ). Discounting relapses, children acquired equal numbers of new *P. falciparum* (*Pf*) and *Pv* blood-stage infections/year (*Pf*-$_{mol}$FOB = 0–18, *Pv*-$_{mol}$FOB = 0–23) resulting in comparable spatial and temporal patterns in incidence and prevalence of infections. Including relapses, *Pv*-$_{mol}$FOB increased >3 fold (relative to PQ-treated children) showing greater heterogeneity at individual (*Pv*-$_{mol}$FOB = 0–36) and village levels. *Pf*- and *Pv*-$_{mol}$FOB were strongly associated with clinical episode risk. Yearly *Pf* clinical incidence rate (IR = 0.28) was higher than for *Pv* (IR = 0.12) despite lower *Pf*-$_{mol}$FOB. These relationships between $_{mol}$FOB, clinical incidence and parasite prevalence reveal a comparable decline in *Pf* and *Pv* transmission that is normally hidden by the high burden of *Pv* relapses.
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Introduction

Renewed emphasis on malaria control has resulted in substantial reductions in overall malaria prevalence and incidence in many endemic countries (***World Health Organization, 2015***). However, where transmission persists, it is highly heterogeneous even on small spatial scales (***Bousema et al., 2012***). Individual exposure is further influenced by factors such as use of bednets, attractiveness to mosquitoes, or behavioural differences. In Papua New Guinea (PNG), malaria prevalence has sharply declined in the last decade, largely as a result of two nationwide distributions of long-lasting insecticide treated bednets (LLIN) (***Hetzel et al., 2015***; ***Hetzel et al., 2014***; ***Hetzel et al., 2012***). *P. vivax* and *P. falciparum* PCR-prevalence in the general population was reduced from 32% and 39% in 2006 to 13% and 18% in 2010 (***Koepfli et al., 2015***). Already before this decline in malaria prevalence, studies in PNG had reported significant heterogeneity in malaria transmission attributed to local population structure and geographical diversity (***Hetzel et al., 2015***; ***Cattani et al., 1986***; ***Genton et al., 1995***; ***Müller et al., 2003***; ***Mueller et al., 2009a***).

eLife digest Malaria is caused by five different species of parasites that are transmitted to humans by bites from parasite-carrying mosquitos. Once in human blood, the parasites rapidly multiply. People who live in countries where malaria is common may become infected and never show any symptoms because their immune systems are able to keep parasite numbers low. Repeated infections, or infection with more than one species of malaria parasite also are common. Some species of malaria, including *Plasmodium vivax*, can hibernate in the liver for weeks or months after the infection and only become active later.

Asymptomatic infections, multi-parasite infections, and reactivating parasites make it hard to measure how often new malaria infections occur. One way scientists can determine if a new infection has occurred is by genotyping the parasites in a person's blood. Genotyping involves looking for small differences in the parasite DNA. For example, a study in Papua New Guinea, where *P. vivax* is very common, showed that reactivations of hibernating parasites were more common than new infections.

Now, Hofmann et al. use the same study in Papua New Guinea to compare the frequency and consequences of new infections with *P. vivax* and another malaria parasite, *Plasmodium falciparum*. In the study, 466 children from 6 villages were followed for 8 months with tests every 2 to 4 weeks to genotype the parasites in their blood. Some of the children were treated with antimalarial drugs to help wipe out any existing parasites including hibernating ones. While *P. vivax* was about twice as common in blood samples—likely due to reactivation—genotyping showed that new infections with the two parasites occur at equal rates and often at the same times and locations.

Hofmann et al. also showed that some villages and some children had much higher rates of infection than others. This difference could not fully be explained by use of bednets or other preventive measures. Children were more likely to become ill from *P. falciparum* than *P. vivax* even though *P. vivax* was more common. But children with more frequent infections with *P. falciparum* seemed better able to manage the parasites and were less likely to develop symptoms that those with infrequent infections. The experiments show that genotyping may help scientists better track new malaria infections and develop better strategies to prevent or treat malaria.

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Prior to the up-scaling of malaria control, *P. vivax* endemicity in PNG was among the highest worldwide (**Hetzel et al., 2015**). Clinical immunity to *P. vivax* was acquired very rapidly in PNG children, and the incidence of *P. vivax* clinical episodes peaked in children younger than two years with only very few *P. vivax* clinical episodes reported in children older than 5 years or adults (**Genton et al., 2008**; **Michon et al., 2007**; **Lin et al., 2010**; **Betuela et al., 2012**). In contrast, the risk for uncomplicated *P. falciparum* clinical episodes increased during early childhood (**Lin et al., 2010**) and significant reductions in incidence of clinical episodes or high-density infections were only observed in children aged 5 years and older (**Michon et al., 2007**). Compared to the incidence of clinical malaria, prevalence of *P. falciparum* and *P. vivax* peaked in older age groups, with asymptomatic infections remaining common until adulthood in PNG (**Koepfli et al., 2015**; **Mueller et al., 2009a**). Concordant with the species-specific pattern in the burden of clinical episodes, *P. vivax* prevalence peaked in younger age groups than *P. falciparum* prevalence (**Koepfli et al., 2015**; **Mueller et al., 2009a**).

As malaria transmission declines, it is important to understand the resulting changes in malaria prevalence and clinical incidence patterns, as well as the extent of heterogeneity in transmission within malaria endemic regions so that high-risk areas can be identified and targeted (**Mosha et al., 2014**). Most attempts to delineate high and low transmission areas made to-date, by both researchers and control programs, have used passive case surveillance or cross-sectional malaria indicator surveys. These surveillance strategies result in clinical incidence and prevalence estimates, both of which are surrogate markers for transmission. A more accurate understanding of the relationship between exposure to new infections and malaria prevalence or clinical incidence is needed to determine how accurately these surrogate markers represent heterogeneity in transmission at local scales. In addition, quantifying clinical incidence in relation to exposure to blood-stage

infections can increase our insight into the development and maintenance of immunity to malaria in a setting of sustained malaria control (**Battle et al., 2015**; **Cameron et al., 2015**).

The molecular force of blood-stage infection ($_{mol}$FOB) describes the number of new genotypes observed in consecutive blood samples from cohort participants over time (**Mueller et al., 2012**; **Koepfli et al., 2013**). Genotyping of highly polymorphic markers detects superinfecting parasite clones in asymptomatic (but parasitaemic) or symptomatic individuals. $_{mol}$FOB thus provides a longitudinal, individual and quantitative measure for exposure to new blood-stage malaria infections (**Mueller et al., 2012**; **Koepfli et al., 2013**). For *P. falciparum*, $_{mol}$FOB is closely linked to the number of infective mosquito bites and therefore is a direct proxy for the actual force of infection (FOI) and thus for transmission in endemic settings (**Smith et al., 2010**). For *P. vivax*, clones appearing in the blood-stream can either originate directly from an infective mosquito bite or from a relapsing liver hypnozoite (**Koepfli et al., 2013**). For *P. vivax*, $_{mol}$FOB is thus a compound measure of exposure to newly acquired infections from mosquito bites and relapsing blood-stage infections.

The usefulness of $_{mol}$FOB as a surrogate marker of individual exposure was validated originally in a cohort of young PNG children 1–4 years of age, in which species-specific $_{mol}$FOB was the most important predictor of clinical incidence for both species (**Mueller et al., 2012**; **Koepfli et al., 2013**). Although some spatial heterogeneity of transmission was observed in that study for both species, due to the high level of transmission the species-specific difference in rate of immune acquisition was the predominant feature in that study. While *P. vivax* $_{mol}$FOB (*Pv*-$_{mol}$FOB) did not change with age, the incidence of *P. vivax* clinical episodes decreased significantly with age, with a faster rate of decrease in children with high *Pv*-$_{mol}$FOB [12]. *P. falciparum* $_{mol}$FOB (*Pf*-$_{mol}$FOB) in that cohort was lower compared to *Pv*-$_{mol}$FOB and the incidence of *P. falciparum* clinical episodes increased in parallel with an increasing *Pf*-$_{mol}$FOB in children 1–3 years, reaching a plateau thereafter (**Lin et al., 2010**; **Mueller et al., 2012**). These earlier results indicated that (i) immunity to *P. vivax* is acquired more rapidly in children with higher cumulative exposure, that (ii) this developing immunity led to proportionally fewer clinical *P. vivax* episodes in older children despite similar exposure to new *P. vivax* blood-stage infections, and that (iii) higher exposure to *P. vivax* blood-stage infections, compared to *P. falciparum*, resulted in a more advanced immunity to *P. vivax* in this age group compared to *P. falciparum* (**Doolan et al., 2009**; **Longley et al., 2016**). The major challenge to these cross-species comparisons lies within the intrinsic differences of *Pf*- and *Pv*-$_{mol}$FOB: whereas

Table 1. Characteristics of study participants by village.

Village	N	% female	Mean age (±SD)	Mean weight (±SD)	% LLIN use at enrolment*	Mean LLIN use during follow-up† (%, range)	Mean Hb (±SD)
Amahup	119	53	7.6 (±1.5)	19.8 (±3.3)	99	99 (50–100)	11.1 (±1.0)
Albinama	99	43	7.7 (±1.5)	20.0 (±3.3)	95	97 (78–100)	11.7 (±1.8)
Balanga	54	59	7.8 (±1.6)	19.8 (±4.3)	96	99 (83–100)	11.3 (±1.1)
Balif	93	51	7.8 (±1.5)	20.3 (±3.3)	91	99 (69–100)	11.7 (±1.2)
Bolumita	70	50	7.4 (±1.7)	19.3 (±2.9)	77	92 (56–100)	10.7 (±1.0)
Numangu	31	55	7.4 (±1.6)	19.2 (±4.6)	100	100 (92–100)	12.1 (±1.4)
Total	**466**	**51**	**7.6 (±1.5)**	**19.8 (±3.5)**	**93**	**100 (50–100)**	**11.4 (±1.4)**

* LLIN use in the night preceding enrolment.

† Information on LLIN use in the previous night was collected at each follow-up visit and averaged across follow-up per participant. Mean LLIN use by village was calculated from the averaged individual LLIN use.

Hb: Haemoglobin.

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Table 2. *Plasmodium* infection status at enrolment by village.

	P. falciparum				*P. vivax*				*P. malariae*			*P. ovale*		
Village	N pos.	Prevalence by qPCR (CI_{95})	% mixed*	Mean† density (IQR)	Mean MOI‡ (range)	N pos.	Prevalence by qPCR (CI_{95})	% mixed*	Mean† density (IQR)	Mean MOI‡ (range)	N pos.	Prevalence by qPCR (CI_{95})	% mixed*	N pos.	Prevalence by qPCR (CI_{95})	% mixed*
Albinama	18	18 (11–27)	72	131 (38–189)	1.4 (1–4)	54	55 (44–65)	24	3 (1–17)	1.8 (1–7)	9	9 (5–17)	67	5	5 (2–12)	100
Amahup	14	12 (7–19)	57	56 (14–105)	1.6 (1–5)	46	39 (30–48)	24	3 (1–29)	2.2 (1–7)	12	10 (6–17)	83	0	0	
Balanga	15	28 (17–42)	67	79 (30–848)	1.7 (1–5)	23	43 (30–57)	43	2 (1–28)	2.0 (1–7)	9	17 (8–30)	56	2	3 (0–14)	50
Balif	8	9 (4–17)	63	64 (10–325)	2.0 (1–4)	35	38 (30–48)	14	2 (1–14)	1.9 (1–6)	7	8 (3–15)	57	0	0	
Bolumita	50	71 (59–81)	80	331 (62–1988)	2.2 (1–8)	47	67 (55–78)	81	3 (2–27)	2.9 (1–10)	28	40 (29–52)	89	8	11 (5–22)	100
Numangu	8	26 (13–45)	75	192 (30–848)	1.1 (1–2)	18	58 (39–75)	28	3 (1–25)	1.6 (1–5)	4	13 (4–31)	50	0	0	
Overall	**113**	**24 (20–28)**	**73**	**163 (20–1103)**	**1.9 (1–8)**	**223**	**48 (43–52)**	**37**	**3 (1–23)**	**2.2 (1–10)**	**69**	**15 (12–18)**	**75**	**15**	**3 (2–5)**	**93**
***p*-value§**		<0.001	0.034	0.086	0.047		<0.001	<0.001	0.947	0.020		<0.001	0.086		<0.001	0.133

* % of infections by qPCR that are mixed-species infections.
† Geometric mean of species-specific *18S rRNA* copy numbers per µl blood.
‡ MOI, multiplicity of infection: number of *Pf-msp2* and *Pv-msp1F3* alleles per infection.
§ Differences between villages were tested for using Chi^2 and Fisher's exact test (prevalence, proportion mixed) or Kruskal-Wallis test (MOI, log_{10}-transformed parasite density).
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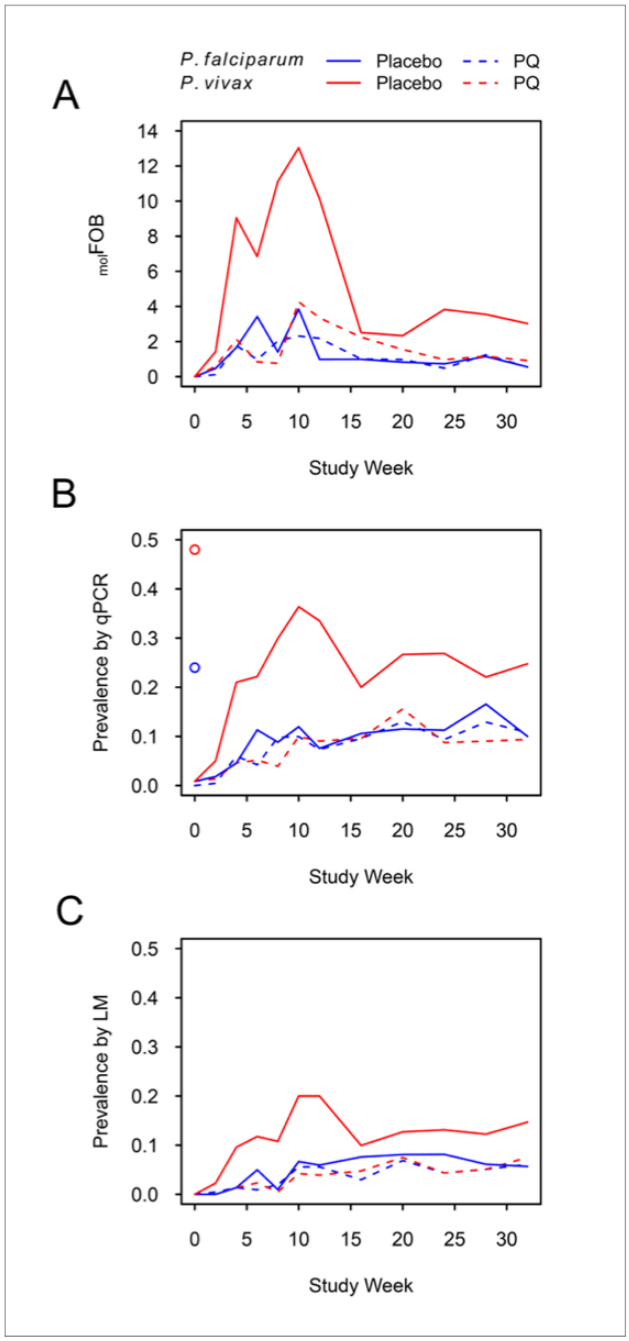

Figure 1. *P. falciparum* and *P. vivax* $_{mol}$FOB (A), prevalence by qPCR (B) and LM (C) by week of follow-up. Blue lines, *P. falciparum*; red lines, *P. vivax*; solid lines, placebo arm; dashed lines, PQ arm. Open circles in (**B**) mark enrolment qPCR prevalence for each species.

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Figure 1 continued on next page

Figure 1 continued

The following figure supplements are available for figure 1:

Figure supplement 1. Definition of new infections for calculating $_{mol}$FOB.

DOI: https://doi.org/10.7554/eLife.23708.006

Figure supplement 2. *P. ovale* and *P. malariae* prevalence by qPCR during follow-up.

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Pf-$_{mol}$FOB is a direct marker of mosquito-borne transmission, *Pv*-$_{mol}$FOB is a composite measure reflecting both newly acquired infections and those caused by relapses of previously acquired infections.

In this study, we extend the analysis of $_{mol}$FOB's relationship with incidence of clinical malaria episodes to older PNG children and a lower transmission scenario. In addition, given the unique study design that randomized blood-stage only or blood- plus liver-stage treatment at enrolment (***Robinson et al., 2015***), we are now, for the first time, able to compare the incidence of newly acquired *P. falciparum* infections with both the incidence of newly acquired *P. vivax* infections and relapsing *P. vivax* infections. Advancing on previous studies that investigated each species individually (***Mueller et al., 2012***; ***Koepfli et al., 2013***), we now provide a comparative analysis of *P. falciparum* and *P. vivax*, directly exploring the role of exposure to multiple *Plasmodium* species in the development of clinical immunity to *P. falciparum* and *P. vivax* malaria. We quantify in detail the extent of heterogeneity in $_{mol}$FOB on a small geographical scale and relate this to heterogeneity in clinical episode incidence to investigate effects of small-scale variation in malaria transmission on local malaria epidemiology. By combining in-depth molecular parasitological data with demographic and clinical data, this study thus provides detailed insights into the changing epidemiology of malaria in PNG in response to intense malaria control efforts.

Table 3. Multivariable predictors for time to recurrent blood-stage infection with *Plasmodium* species by qPCR

	P. vivax			*P. falciparum*			*P. malariae*			*P. ovale*		
Variable	**AHR***	**CI$_{95}$**	***p*-value**	**AHR***	**CI$_{95}$**	***p*-value**	**AHR***	**CI$_{95}$**	***p*-value**	**AHR***	**CI$_{95}$**	***p*-value**
PQ treatment	0.18	0.13–0.25	<0.001	0.73	0.52–1.02	0.064	0.51	0.22–1.19	0.121	0.31	0.12–0.75	0.010
Age	0.95	0.87–1.04	0.247	1.05	0.94–1.17	0.361	0.98	0.75–1.29	0.905	0.96	0.74–1.26	0.793
LLIN use at enrolment	0.62	0.39–0.98	0.043	0.84	0.49–1.44	0.531	1.33	0.33–6.09	0.715	0.95	0.26–3.43	0.936
Hb at enrolment (g/dl)	0.88	0.80–0.98	0.019	0.90	0.80–1.02	0.099	0.83	0.61–1.12	0.224	0.92	0.66–1.28	0.634
Village												
Albinama (ref)	1			1			1			1		
Amahup	0.45	0.29–0.71	0.001	0.58	0.31–1.11	0.101	0.34	0.07–1.79	0.205	2.83	0.29–27.48	0.370
Balanga	2.15	1.40–3.31	<0.001	1.81	0.99–3.30	0.054	0.92	0.24–3.60	0.910	7.74	0.85–70.45	0.070
Balif	1.00	0.66–1.54	0.983	0.60	0.30–1.19	0.145	0.24	0.03–2.07	0.193	4.60	0.51–41.41	0.173
Bolumita	3.34	2.09–5.33	<0.001	4.73	2.69–8.30	<0.001	1.21	0.34–4.31	0.770	19.43	2.19–172.37	0.008
Numangu	0.83	0.44–1.59	0.583	2.29	1.17–4.50	0.015	0.82	0.15–4.53	0.823	3.17	0.19–52.41	0.420
Infection status at enrolment (by qPCR)												
Uninfected (ref)	1			1			1			1		
P. vivax	1.27	0.91–1.78	0.165	1.37	0.86–2.20	0.186	0.92	0.20–4.18	0.913	2.17	0.68–6.97	0.192
P. falciparum	1.36	0.84–2.19	0.205	1.56	0.86–2.82	0.145	3.54	0.85–14.72	0.083	1.25	0.26–5.90	0.779
P. malariae	0.83	0.38–1.85	0.655	0.99	0.38–2.56	0.977	6.35	1.31–30.81	0.022	1.58	0.17–14.30	0.676
Mixed *P.f. or P.v.*†	1.74	1.14–2.65	0.010	2.08	1.25–3.48	0.005	3.37	0.88–12.90	0.076	2.03	0.55–7.53	0.287

* AHRs were modeled using Cox proportional hazard regression.

† Mixed infection including *P. falciparum* or *P. vivax* infection in conjunction with one or more other *Plasmodium spp.*

PQ: Primaquine; LLIN: long-lasting insecticide-treated net; Hb: haemoglobin.

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Results

Demographic and parasitological parameters at enrolment

This study was conducted in six villages in Maprik district, East Sepik Province, PNG between August 2009 and May 2010 (**Robinson et al., 2015**). 524 children aged 5–10 years were enrolled and randomized to receive either chloroquine (CQ), artemeter-lumefantrine (AL) and primaquine (PQ); or CQ, AL and placebo. Demographic parameters of the 466 children that completed the full course of randomized treatment with PQ/CQ/AL (n = 233) or placebo/CQ/AL (n = 233), and were thereafter closely followed for 8 months, were comparable between the six villages (**Table 1**).

P. vivax was the most common infection at enrolment with 48% of children positive by quantitative PCR (qPCR), followed by *P. falciparum* (24%), *P. malariae* (15%) and *P. ovale* (3%; **Table 2**). 39% of children were not infected with any *Plasmodium* species at enrolment. The vast majority of *P. malariae* (75%) and almost all *P. ovale* infections (93%) occurred in children co-infected with either *P. vivax* and/or *P. falciparum* (**Table 2**). Prevalence of each *Plasmodium* species varied between villages (*P. falciparum*, 9–71%; *P. vivax*, 38–67%; *P. malariae*, 8–40%; *P. ovale*, 0–11%; **Table 2**) and was highest in Bolumita for all species. Accordingly, mixed-species infections were also most prevalent in Bolumita (**Table 2**). The multiplicity of infection (MOI), that is, the number of parasite genotypes per infection, also varied between villages for both species (mean *P. falciparum* MOI, 1.1–2.2 clones/infection; mean *P. vivax* MOI, 1.6–2.9 clones/infection) and children from Bolumita carried more multi-clone infections with *P. vivax* and *P. falciparum* than children in other villages (**Table 2**). Mean *P. falciparum* parasite density was almost two- to six-fold higher in Bolumita (331 18S rRNA gene copies/µl) than in other villages (56–192 18S rRNA gene copies/µl, **Table 2**).

$_{mol}$FOB and parasite prevalence after randomized radical cure treatment

Children who had received PQ for clearance of *P. vivax* hypnozoites experienced similar numbers of new blood-stage infections with *P. falciparum* and *P. vivax* during follow-up (mean *Pf*-$_{mol}$FOB = 1.5

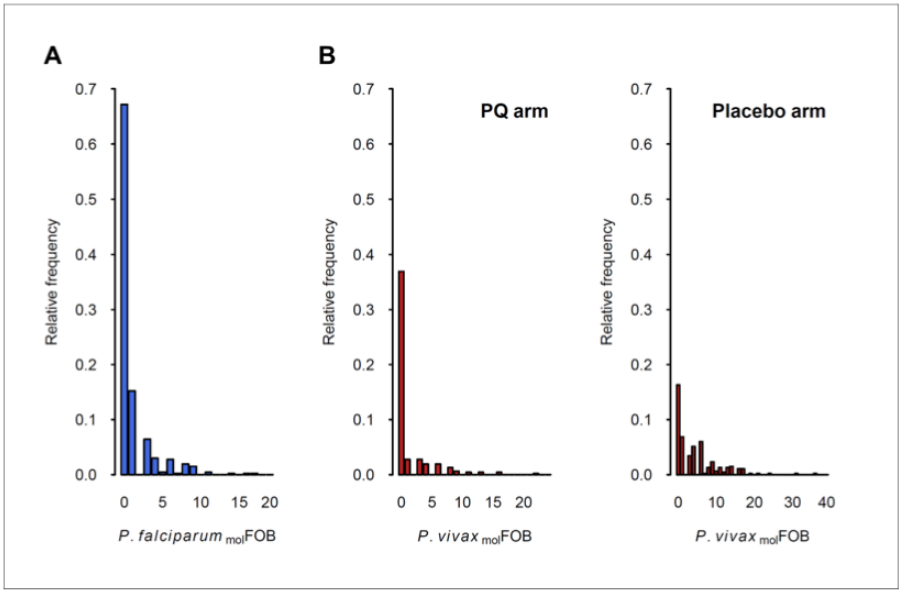

Figure 2. Distribution of *P.falciparum* $_{mol}$FOB (**A**) and *P. vivax* $_{mol}$FOB by treatment arm (**B**). Relative frequencies among the 466 children are shown.

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CI_{95} [1.3–1.7] new blood-stage clones/year, *Pv*-$_{mol}$FOB = 1.6 [1.4–1.9] new blood-stage clones/year, ***Figure 1A***, ***Figure 1—figure supplement 1***). *Pf*-$_{mol}$FOB in the placebo arm was comparable to the PQ arm (mean *Pf*-$_{mol}$FOB = 1.4 [1.2–1.6] new blood-stage clones/year), whereas due to the hypnozoite reservoir *Pv*-$_{mol}$FOB was more than three times higher in the placebo arm compared to the PQ arm (mean *Pv*-$_{mol}$FOB = 5.4 [4.9–5.8] new blood-stage clones/year). *Pv*-$_{mol}$FOB in the placebo arm showed a pronounced peak at months 2–3 of follow-up, which likely represents a wave of fast-relapsing hypnozoites in children who did not receive PQ (***Figure 1A***).

P. vivax prevalence in the PQ arm was comparable to *P. falciparum* prevalence throughout the study and increased steadily, irrespective of the diagnostic method used (***Figure 1B and C***). *P. vivax* prevalence increased more rapidly in the placebo arm until month 3 of follow-up and dropped thereafter, similar to patterns in *Pv*-$_{mol}$FOB in the same arm. Prevalence as measured by qPCR did not reach pre-treatment levels until the end of the study for any of the four *Plasmodium* species (***Figure 1B***, ***Figure 1—figure supplement 2***).

At the end of follow-up, *P. vivax* prevalence by qPCR in the placebo arm was 25% [19–31%], and therefore more than two-fold higher than in the PQ arm (9% [6–14%]; ***Figure 1B***). Also, throughout follow-up, *P. vivax* prevalence in the placebo arm was 2–3 fold higher compared to the PQ arm, suggesting that at least 50% of the overall *P. vivax* prevalence in this cohort can be attributed to the contribution of relapses.

Similarly, throughout and at the end of follow-up *P. vivax* prevalence in the placebo arm was 2–3 fold higher compared to *P. falciparum* (irrespective of treatment arm; *P. falciparum* prevalence at

Table 4. Multivariable predictors of *Pv*- and *Pf*-$_{mol}$FOB per follow-up interval.
Model predictions from this model were used for mapping $_{mol}$FOB in ***Figure 3A***.

	P. vivax						*P. falciparum*		
	PQ arm			Placebo arm			Combined arms		
Variable	IRR*	CI_{95}	*p*-value	IRR*	CI_{95}	*p*-value	IRR*	CI_{95}	*p*-value
PQ treatment	n.a.†	n.a.	n.a.	n.a.	n.a.	n.a.	0.89	0.65–1.22	0.474
New *P. falc.* infections in interval‡	1.32	0.92–1.89	0.134	1.10	0.85–1.42	0.466	n.a.	n.a.	n.a.
New *P. vivax* infections in interval‡	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	1.15	0.97–1.36	0.100
Age	0.86	0.74–1.01	0.059	0.95	0.87–1.04	0.305	1.03	0.92–1.14	0.640
LLIN use at enrolment	0.96	0.51–1.79	0.897	0.62	0.43–0.91	0.013	1.07	0.7–1.62	0.755
Hb at enrolment (g/dl)	0.85	0.72–1.01	0.063	0.91	0.85–0.99	0.025	0.85	0.75–0.97	0.013
Village									
Albinama (ref)	1			1			1		
Amahup	0.02	0–0.11	<0.001	0.56	0.34–0.91	0.020	0.52	0.25–1.07	0.074
Balif	0.85	0.4–1.8	0.664	1.74	1.16–2.61	0.007	1.81	0.98–3.35	0.059
Balanga	0.28	0.1–0.82	0.020	1.13	0.73–1.73	0.590	0.75	0.37–1.52	0.423
Bolumita	1.52	0.73–3.17	0.268	2.67	1.83–3.9	<0.001	6.05	3.32–11.05	<0.001
Numangu	0.5	0.15–1.68	0.264	0.76	0.4–1.43	0.394	2.8	1.39–5.64	0.004
Study Day									
Day 0–35 (ref)	1			1			1		
Day 36–80	1.37	0.54–3.48	0.509	1.99	1.39–2.84	<0.001	2.42	1.44–4.07	0.001
Day 81–175	1.34	0.57–3.12	0.503	0.89	0.61–1.3	0.538	1.13	0.7–1.84	0.616
Day > 175	0.65	0.25–1.69	0.374	0.56	0.38–0.83	0.004	0.87	0.48–1.56	0.643

*IRRs were modeled per sampling interval using negative binomial generalized estimating equations allowing for repeated visits with log-link and an exchangeable correlation structure.

† n.a., not applicable.

‡$_{mol}$FOB in the follow-up interval (time-varying covariate).

PQ: Primaquine; LLIN: long-lasting insecticide-treated net; Hb: haemoglobin.

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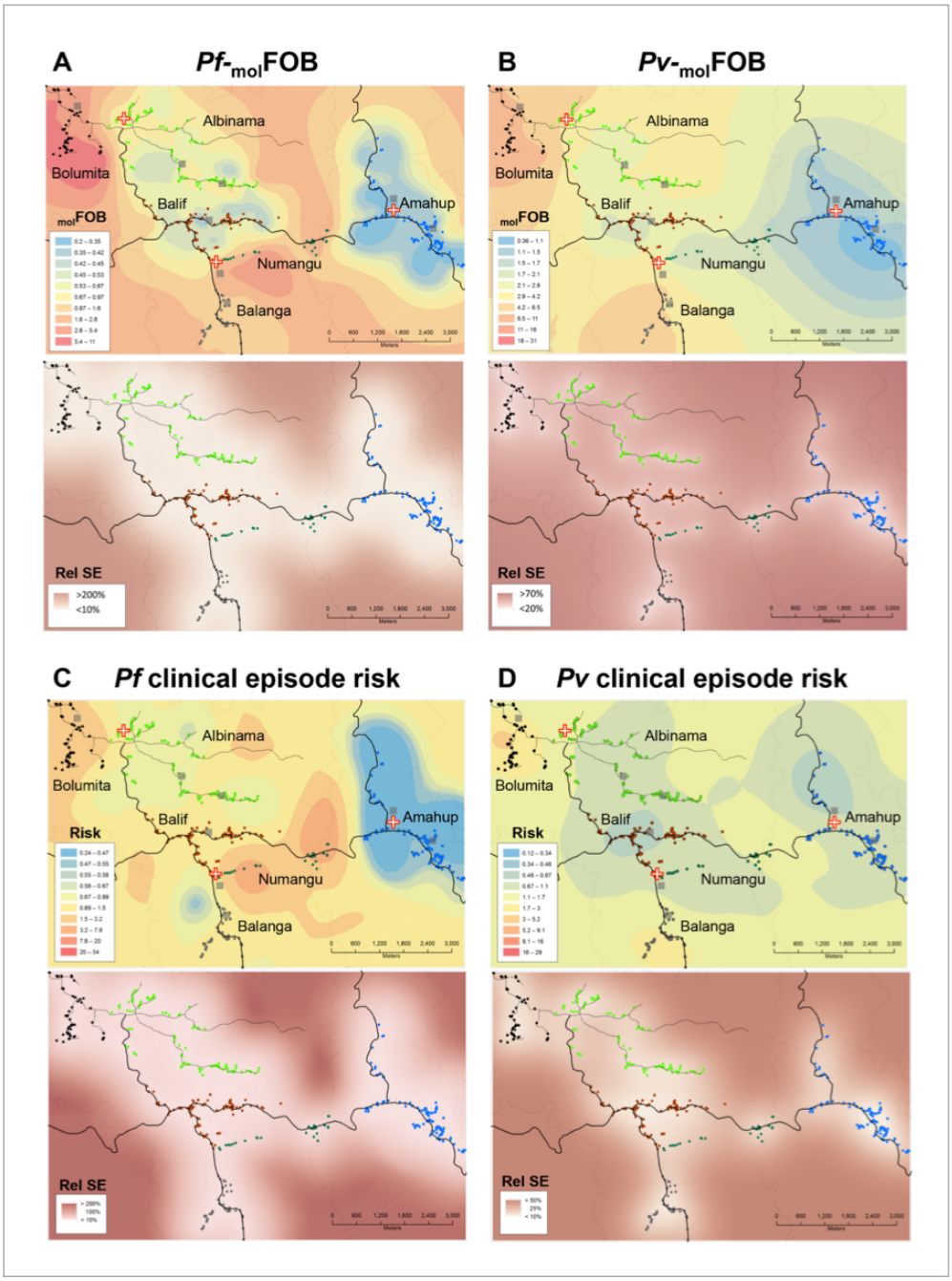

Figure 3. Heterogeneity in $_{mol}$FOB (**A, B**) and clinical episode risk (**C, D**) of *P.falciparum* (**A, C**) and *P. vivax* (**B, D**). Upper panels show the kriging fit of model predictions of $_{mol}$FOB and clinical episode risk of children in both treatment arms. Lower panels show the standard error relative to the kriging estimate. Dots represent study participants' houses and are color-coded according to village. Black lines: vehicle-accessible road; dark grey lines:

Figure 3 continued on next page

Figure 3 continued

vehicle-inaccessible road; light grey lines: river; red/white cross: health center or aid post; grey square: school or enrolment location. Maps were prepared using ArcGIS 10.2 (Esri, USA).

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end of follow-up, 10% [8–14%]), which is in agreement with the prevalence pattern at enrolment. Assuming equal transmission from mosquitoes for both species, which was corroborated by a comparable *Pf*-$_{mol}$FOB and *Pv*-$_{mol}$FOB in the PQ arm, *P. vivax* relapses have contributed to a *P. vivax* prevalence twice as high as that of *P. falciparum*.

Risk of recurrent blood-stage infections and $_{mol}$FOB during follow-up

In the present study design, recurrent blood-stage infection can either originate from a new transmission event (both arms and all species) or for *P. vivax* and *P. ovale* also from a relapse of any previous infection (placebo arm only). After adjusting for the effect of PQ treatment (**Robinson et al., 2015**), village of residence and infection status by qPCR at enrolment were the main predictors for the risk of recurrent *Plasmodium spp.* during follow-up (**Table 3**). Interestingly, in addition to a protective effect against recurrent *P. vivax* and *P. ovale*, the risk of recurrent *P. falciparum* was also reduced by 27% [0–48%] after PQ treatment (p=0.064).

The risk of a recurrent infection (measured by qPCR) with *P. falciparum*, *P. vivax* and *P. ovale* varied more than 7-fold between villages, with a higher risk observed in Bolumita (78%, 77%, and 15% with recurrent *P. vivax*, *P. falciparum* and *P. ovale*, respectively) compared to the other villages (recurrent *P. vivax*, range 25–73%; recurrent *P. falciparum*, range 12–44%; recurrent *P. ovale*, range 0–7%). For *P. falciparum* and *P. vivax*, a mixed infection at enrolment as measured by qPCR was further associated with up to a two-fold increased risk of recurrent infection (*P. falciparum*: AHR = 2.08 [1.25–3.48], p=0.005; *P. vivax*: AHR = 1.74 [1.14–2.65], p=0.010; **Table 3**), supporting the idea that focal transmission within villages leads to the presence of high-risk and low-risk individuals. For *P. malariae*, the infection status at enrolment was a stronger predictor of risk of recurrent infection than village of residence. An infection with *P. falciparum*, *P. malariae* or a mixed infection at enrolment as measured by qPCR was associated with up to a 6-fold increase in risk of recurrent *P. malariae* ($AHR_{Pf\text{-}enrol}$ = 3.54 [0.85–14.72], p=0.083; $AHR_{Pm\text{-}enrol}$ = 6.35 [1.31–30.81], p=0.022; AHR_{mixed} = 3.37 [0.88–12.90], p=0.076; **Table 3**).

Reported use of a LLIN during the night previous to enrolment was associated with a reduced risk of recurrent *P. vivax* and *P. falciparum* in univariate analyses (**Supplementary file 1** - Table 1) but to a lesser extent in multivariable analyses (*P. vivax*: AHR = 0.62 [0.39–0.98], p=0.043, *P. falciparum*: AHR = 0.84 [0.49–144], p=0.531). Haemoglobin (Hb) level at enrolment was negatively associated with the risk of recurrent infection with *P. vivax* (AHR = 0.88 [0.80–0.98], p=0.019) and *P. falciparum* (AHR = 0.90 [0.80–1.02], p=0.099). Patterns in the risk of recurrent infections with *P. falciparum* and *P. vivax* as measured by light microscopy (LM, **Supplementary file 2**) were similar to those observed for re-infection as measured by qPCR. When based on LM observation (but not as measured by qPCR), increasing age was associated with a reduced risk of recurrent *P. vivax* (AHR = 0.85 [0.77–0.95], p=0.004; **Supplementary file 2**) but an increased risk of recurrent *P. falciparum* (AHR = 1.16 [1.01–1.33], p=0.037; **Supplementary file 2**).

The incidence of new *P. falciparum* and *P. vivax* blood-stage clones detected during follow-up, that is $_{mol}$FOB, was highly variable between individual children and ranged from 0 to 18 new clones/year for *P. falciparum* (**Figure 2A**) and 0 to 36 or 23 new blood-stage clones/year for *P. vivax* in the placebo or PQ arm, respectively (**Figure 2B**). Mean *Pf*- and *Pv*-$_{mol}$FOB varied significantly between villages and were higher in Bolumita (*Pf*-$_{mol}$FOB = 4.9 new blood-stage clones/year, *Pv*-$_{mol}$$FOB_{PQ\ arm}$=4.4 new blood-stage clones/year, *Pv*-$_{mol}$$FOB_{placebo\ arm}$=12.1 new blood-stage clones/year; **Table 4**; **Figure 3**) than in the other villages (*Pf*-$_{mol}$FOB, range 0.7–1.8 new blood-stage clones/year; *Pv*-$_{mol}$$FOB_{PQ\ arm}$, range 0.03–2.2 new blood-stage clones/year; *Pv*-$_{mol}$$FOB_{placebo\ arm}$, range 2.3–7.4 new blood-stage clones/year). In univariate analyses, new *P. vivax* infections were strongly associated with new *P. falciparum* infections per sampling interval and vice versa, suggesting concurrent exposure to the two species (**Supplementary file 1** – Table 2). However, these effects were reduced when other variables of varying exposure such as village of residence or infection at enrolment were

Table 5. Multivariable predictors for time to *P. vivax* and *P. falciparum* clinical episodes.
Model predictions from this model were used for mapping the relative risk of clinical malaria episodes in ***Figure 3C and D***.

	P. vivax			*P. falciparum*		
Variable	**AHR***	**CI95**	***p*-value**	**AHR***	**CI95**	***p*-value**
PQ treatment	0.76	0.34–1.68	0.497	1.79	1.05–3.03	0.031
P. vivax $_{mol}$FOB‡	1.07	1.04–1.09	<0.001	n.a.	n.a.	n.a.
P. falciparum $_{mol}$FOB‡	n.a.	n.a.	n.a.	1.15	1.11–1.21	<0.001
Age	0.62	0.46–0.84	0.002	0.98	0.85–1.13	0. 799
LLIN use at enrolment	0.84	0.24–2.88	0.778	0.44	0.22–0.87	0.018
Hb at enrolment (g/dl)	0.95	0.74–0.67	0.668	0.85	0.71–1.01	0.070
Village						
Albinama (ref)	1			1		
Amahup	0.89	0.23–3.46	0.871	0.65	0.20–2.08	0.465
Balif	1.48	0.45–4.86	0.518	1.26	0.50–3.14	0.626
Balanga	0.85	0.21–3.53	0.827	1.39	0.59–3.30	0.455
Bolumita	0.99	0.24–4.03	0.987	1.32	0.58–3.03	0.508
Numangu	1.00	0.23–4.31	0.997	4.29	2.06–8.97	<0.001
Infection status at enrolment (by qPCR)						
Uninfected (ref)	1			1		
P. vivax	0.77	0.29–2.07	0.608	1.64	0.91–2.95	0.101
P. falciparum	1.74	0.59–5.11	0.316	0.97	0.34–2.77	0.954
Mixed *P.f. or P.v.*	1.59	0.56–4.50	0.381	1.24	0.57–2.68	0.582

* AHRs were modeled using multiple failure Cox proportional hazard regression.
† n.a., not applicable
‡ Average $_{mol}$FOB until the time of failure (time-varying covariate).
PQ: Primaquine; LLIN: long-lasting insecticide-treated net; Hb: haemoglobin.
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included in the multivariable model (*P. vivax*: $IRR_{PQ\ arm}$=1.32 [0.92–1.89], p=0.134; $IRR_{Placebo\ arm}$=1.10 [0.85–1.42], p=0.466; *P. falciparum*: IRR = 1.15 [0.97–1.36], p=0.100, ***Table 4***). LLIN use,

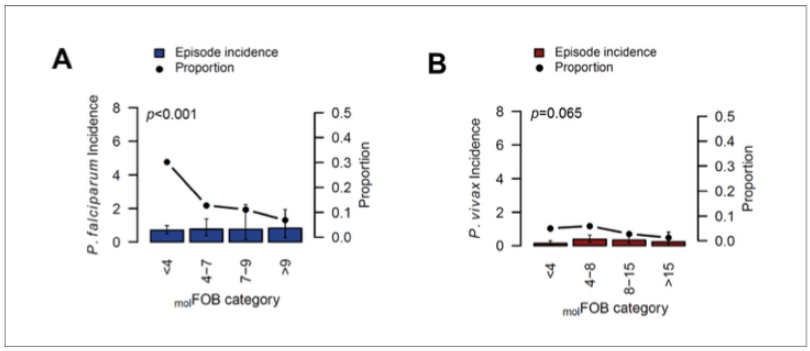

Figure 4. The incidence of *P.falciparum* (**A**) and *P. vivax* (**B**) clinical episodes relative to $_{mol}$FOB. Mean clinical episode incidence is shown as bars (left axis) and proportion of clinical episode incidence divided by $_{mol}$FOB as connected dots (right axis). Error bars represent 95% CIs. *p*-values refer to the differences between groups in the proportion of clinical episodes and new infections, assessed by Chi^2 or Fisher's exact test.
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Table 6. Multivariable predictors for odds of *P. falciparum* clinical episodes

	***P. falciparum* episode**		
Variable	**OR***	**CI_{95}**	***p*-value**
PQ treatment	1.42	0.80–2.52	0.226
P. vivax qPCR positive†	0.35	0.15–0.78	0.011
P. falciparum $_{mol}$FOB‡	1.21	1.10–1.34	<0.001
Age	0.93	0.80–1.09	0.370
LLIN at enrolment	0.37	0.16–0.83	0.016
Hb (g/dl) at enrolment	0.88	0.70–1.11	0.292
Village			
Albinama (ref)	1		
Amahup	0.41	0.12–1.39	0.154
Balif	0.9	0.26–3.08	0.870
Balanga	1.19	0.42–3.39	0.747
Bolumita	1.48	0.42–5.17	0.540
Numangu	4.17	1.64–10.58	0.003
Study Day			
Day 0–80	1		
Day 81–175	0.99	0.51–1.91	0.972
Day > 175	0.83	0.39–1.75	0.629

* ORs were modeled using a binomial generalized estimating equation with logit link function using an exchangeable correlation structure.

† Determined as *P. vivax* positive at the same or previous sampling visit.

‡$_{mol}$FOB in the follow-up interval (time-varying covariate).

PQ: Primaquine; LLIN: long-lasting insecticide-treated net; Hb: haemoglobin.

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although strongly associated with lower *Pf*- and *Pv*-$_{mol}$FOB in univariate analyses (***Supplementary file 1*** – Table 2), remained significantly associated in multivariable models only for *P. vivax* in the placebo arm, where sleeping under a LLIN in the night previous to enrolment was associated with a 38% [9–57%] reduction in *Pv*-$_{mol}$FOB (p=0.013, ***Table 4***). Each additional year of age was associated with a 14% [0–26%] reduction *Pv*-$_{mol}$FOB per sampling interval in the PQ arm (p=0.059), while no age effect was observed in the placebo arm or for *P. falciparum* (***Table 4***). Hb level at enrolment was negatively associated with *Pf*- and *Pv*-$_{mol}$FOB (*P. vivax*: $IRR_{PQ\ arm}$=0.85 [0.72–1.01], p=0.063; $IRR_{Placebo\ arm}$=0.91 [0.85–0.99], p=0.025; *P. falciparum*: IRR = 0.85 [0.75–0.97], p=0.013), suggesting anaemia in individuals continuously exposed to blood-stage infections.

Patterns in the risk of *P. vivax* and *P. falciparum* clinical episodes

A total of 98 clinical malaria episodes, here defined as fever plus presence of LM-detectable parasites, were observed during the study period. Of these, 64 (65%) exceeded the previously established pyrogenic thresholds of 2500 and 500 parasites/µl per LM for *P. falciparum* and *P. vivax*, respectively (***Mueller et al., 2009b***). *P. falciparum* was the most common cause of clinical malaria episodes (*P. falciparum*, 64 clinical episodes; *P. vivax*, 31 clinical episodes; mixed *P. falciparum/P. vivax* by LM, 3 clinical episodes), despite lower incidence of new *P. falciparum* blood-stage clones compared with *P. vivax* (*P. falciparum*, 342 new *P. falciparum* blood-stage clones; *P. vivax*, 849 new blood-stage clones). Including clinical episodes with mixed infection as determined by LM in the estimates for both species, clinical incidence rate (IR) was 0.28 [0.21–0.35] *P. falciparum* episodes/year and 0.12 [0.08–0.17] *P. vivax* episodes/year. At least one new blood-stage clone was detected in 70% (47/67) of samples from *P. falciparum* and 71% (24/34) of samples from *P. vivax* clinical episodes. Of these clinical episodes with new blood-stage clones, 96% (45/47) and 83% (20/24) carried only the new but no persistent *P. falciparum* and *P. vivax* clones, respectively.

P. vivax clinical episodes occurred mainly in the placebo arm shortly after directly observed treatment (DOT) (**Robinson et al., 2015**), the time of peak *Pv*-$_{mol}$FOB due to relapsing hypnozoites (**Figure 1A**). On an individual level, *Pv*-$_{mol}$FOB was positively associated with the risk of clinical episodes and each additional blood-stage *P. vivax* clone increased the risk of experiencing a *P. vivax* clinical episode slightly (AHR = 1.07 [1.04–1.09], p<0.001; **Table 5**). No significant differences in *P. vivax* clinical episode risk were observed between villages after adjusting for individual $_{mol}$FOB. The risk for a *P. vivax* clinical episode decreased significantly with age (AHR = 0.62 [0.46–0.84], p=0.002; **Table 5**). This was paralleled by a decrease in *P. vivax* densities with age (by qPCR, exp(β)=0.90 [0.83–0.98], p=0.016; **Supplementary file 3**) indicative of more advanced immunity against *P. vivax* and thus better control of *P. vivax* densities in older children.

Patterns in the occurrence of *P. falciparum* clinical episodes during follow-up were more complex. Between-village variation in *P. falciparum* clinical episode risk remained significant even after adjusting for individual exposure. This effect was mainly apparent in Numangu, where children were at three- to six-fold higher risk for clinical episodes than children in other villages (Numangu AHR = 4.29 [2.06–8.97], other villages range AHR = 0.65 [0.20–2.08] to 1.39 [0.59–3.30]; **Table 5**). Overall, *Pv*-$_{mol}$FOB was positively associated with the risk of clinical episodes and each additional *P. falciparum* blood-stage clone slightly increased the risk for *P. falciparum* clinical episodes (AHR = 1.15 [1.11–1.21], p<0.001; **Table 5**); however, relative to the number of new blood-stage clones, *P. falciparum* clinical episodes were less frequent in highly exposed children compared to low-exposed children (**Figure 4**). One clinical episode per three new blood-stage clones was detected in the least exposed children (*Pf*-$_{mol}$FOB <4 new blood-stage clones/year), but only one clinical episode per 15 blood-stage clones in the highest exposed children (*Pf*-$_{mol}$FOB >9 new blood-stage clones/year, Fisher's exact test p<0.001). Age was not associated with the risk of *P. falciparum* clinical episodes. LLIN use at enrolment was associated with a 56% [13–78%] reduced risk of *P. falciparum* clinical episodes (p=0.018; **Table 5**).

A higher risk for *P. falciparum* clinical episodes in children that had received PQ treatment for clearance of *P. vivax* liver stages was observed (AHR = 1.79 [1.05–3.03], p=0.031; **Table 5**), suggesting a potential protective effect of *P. vivax* infections against *P. falciparum* clinical episodes. Analysis to further explore this revealed that a concurrent or recent infection (i.e., at the same or preceding follow-up visit) with *P. vivax* reduced the odds of a *P. falciparum* clinical episode by 65% [22-85%] (p=0.011; **Table 6**). Further indications for a potential interaction between the two species was also observed when analyzing *P. falciparum* parasite densities, which were reduced by 55% [19–75%] (p=0.008; **Supplementary file 3**) in mixed *P. falciparum/P. vivax* infections compared to *P. falciparum* single infections, indicative of suppression of one of the species in mixed infections.

Discussion

In the present study, we describe striking heterogeneity in malaria transmission not only between closely neighboring communities in Maprik district, PNG, but also substantial differences in exposure between individual children from the same village. On village level this heterogeneity is apparent both when using traditional markers such as prevalence of infection, as well as when using the novel 'reference standard' marker of individual exposure $_{mol}$FOB. The increased resolution provided by $_{mol}$FOB further allows quantifying heterogeneity in exposure to new blood-stage infections between individual children. Extending an earlier study in a neighboring area in which younger children had been enrolled, and that had identified $_{mol}$FOB as the most important predictor of malaria clinical episodes (**Mueller et al., 2012**; **Koepfli et al., 2013**), we confirmed that $_{mol}$FOB remains significantly associated with the risk for clinical episodes, but other factors such as age (*P. vivax*), or a mixed *Pf/Pv* infection and village factors not captured by any of the other parameters assessed (*P. falciparum*) have a stronger effect on the risk for clinical malaria (**Table 5 and 6**).

Malaria transmission is often estimated by investigating the more accessible human host rather than the mosquito vector (**Tusting et al., 2014**). Because *P. falciparum* blood-stage infections are a direct outcome of mosquito-to-human transmission, infection parameters assessed in the human blood closely reflect *P. falciparum* transmission. In contrast, relapses arising from dormant hypnozoites contribute substantially to *P. vivax* blood-stage infections (**Robinson et al., 2015**), thus complicating the assessment of mosquito-to-human *P. vivax* transmission via infection parameters measured in the human blood. The unique design of this study, that combined clearance of

hypnozoites in half of the study participants with subsequent measurement of *Pv*-$_{mol}$FOB, allowed us to identify the burden of *P. vivax* infections due to mosquito-to-human transmission (in hypnozoite-cleared individuals) and compare it to the total burden of *P. vivax* infections. We found a highly similar incidence and comparable temporal and spatial heterogeneity of *P. falciparum* and *P. vivax* infection acquired through renewed exposure to infected mosquito bites. In children that experienced the full burden of relapses we found two-fold higher *P. vivax* infection prevalence and 4-times higher incidence ($_{mol}$FOB) compared to *P. falciparum*. This first quantitative comparative assessment of *P. falciparum* and *P. vivax* transmission using non-entomological molecular parameters thus indicates that the observed differences in epidemiology between the two species are largely due to the high burden of relapsing *P. vivax* blood-stage infections (**Robinson et al., 2015**). Our molecular results thus support recent entomological data from Dreikikir district, 50 km from Maprik in East Sepik Province (**Reimer et al., 2016**) as well as earlier studies in East Sepik (**Hii et al., 2001**) that found similar sporozoite rates for *P. falciparum* and *P. vivax*.

Our previous analysis of this cohort had investigated the contribution of the hypnozoite reservoir to *P. vivax* infection and disease in order to inform strategies for achieving a sustained reduction of the *P. vivax* burden in PNG (**Robinson et al., 2015**). Here, we now describe the post-treatment re-infection dynamics in higher temporal detail. Through a detailed comparison of these patterns for *P. vivax* and *P. falciparum* in PQ and placebo-treated children we further elucidate the contribution of relapses to *P. vivax* prevalence and clinical incidence, which are the most commonly used parameters for planning and monitoring of malaria control strategies. *P. vivax* relapses accounted for more than half of the observed *P. vivax* prevalence in this cohort, which is lower than what was previously estimated as the contribution of relapses towards *Pv*-$_{mol}$FOB by comparison of treatment arms (77%, **Robinson et al., 2015**). This difference can be accounted for by the higher number of *P. vivax* multiple clone infections that will accumulate more rapidly in the placebo-arm, where additional parasite clones from relapses and/or new infections may overlap with without a corresponding change in overall prevalence.

While we previously described a sustained effect of PQ treatment with significant reductions in *Pv*-$_{mol}$FOB observed up to eight months post treatment (**Robinson et al., 2015**), here, we describe temporal variation in relapse rate with a rapid and wave-like recurrence of *P. vivax* in children from the placebo arm, who had retained their hypnozoites (**Figure 1A**). The concurrent, modest peaks in *Pf*-$_{mol}$FOB and *Pv*-$_{mol}$FOB in the PQ arm represent seasonal variation in transmission, which is highest in December and January in the study area (**Mueller et al., 2012**); corresponding to weeks 8–14 of follow-up). A much higher peak and subsequent drop in appearance of new clones within three months after blood-stage only treatment, which was mirrored by a corresponding peak and drop in *P. vivax* prevalence (**Figure 1B and C**), suggests that the incidence of relapse infections in the blood was not constant during follow-up. *P. vivax* infections are often observed following treatment of *P. falciparum* malaria (**Douglas et al., 2011**), and it can thus been hypothesized that the frequency of relapses may be temporarily increased after blood-stage antimalarial treatment (**White and Imwong, 2012**). It is thus conceivable that the blood-stage antimalarial at baseline either triggered *P. vivax* relapses directly, or indirectly by allowing more hypnozoites to establish blood-stage infections in parasite-free hosts. Alternatively, blood-stage *P. vivax* infections from hypnozoites relapsing shortly after baseline treatment (during a period when antimalarial drugs were present at sub-curative levels) may be suppressed to sub-detectable densities until complete waning of drug levels, resulting in simultaneous proliferation and detection of many new blood-stage clones within the first weeks after treatment (**Douglas et al., 2011**; **Tarning et al., 2014**). More detailed modeling of the dynamics of individual *P. vivax* blood-stage infections and their association with potential triggers such as treatment or febrile illness will be required to determine the existence and importance of proposed relapse-triggers.

Malaria transmission showed high micro-spatial heterogeneity with more than 10-fold differences in *Pf*- and *Pv*-$_{mol}$FOB (in the PQ arm) between villages despite an overall high LLIN use by the study participants (during follow-up; village average use,>90%; individual use,>50%). Individual LLIN use at enrolment was nevertheless associated with a reduced risk of recurrent *P. falciparum* and *P. vivax* in univariate analyses. However, after adjustment for other related variables (i.e., village of residence or infection status) this association became non-significant. Children living in Bolumita, where both *P. falciparum* and *P. vivax* $_{mol}$FOB and prevalence were highest, had a modestly lower LLIN use (mean during follow-up, 92%; at enrolment, 77%) compared to children from other villages (mean

during follow-up, 97–100%; at enrolment, 91–100%). It is conceivable that LLIN use in the Bolumita community may be less effective in reducing malaria transmission (**Killeen et al., 2007**; **Smith et al., 2009**). Potential differences between villages in mosquito density, behavior, sporozoite rate, proximity of house or play areas to mosquito breeding sites, or human behavioral factors (related to LLIN use or other risk factors) are however likely to be more important determinants for exposure to infective bites. Small-scale variations in vector species and distribution between and within villages in PNG have been described previously (**Cattani et al., 1986**; **Reimer et al., 2016**; **Charlwood et al., 1986**; **Hii et al., 1997**; **Burkot et al., 1988**) and likely account in a large part for the micro-geographic heterogeneity in malariological parameters observed in this and other studies.

Assessing the incidence of new infections from consecutive blood samples using molecular methods (as is necessary to determine $_{mol}$FOB), is complicated by fluctuating densities of clonal parasitemia that may temporarily fall below the limit of detection of the genotyping PCR, leading to imperfect detectability of clones (**Bretscher et al., 2010**; **Felger et al., 2012**; **Koepfli et al., 2011**). For *P. falciparum*, periodical sequestration of clones and absence from the peripheral blood at time of sampling may further contribute to imperfect detectability. For *P. vivax*, generally low parasite densities aggravate the problem of imperfect detectability, and dis- and re-appearance of clones may be a result of imperfect detectability or relapsing hypnozoites. The overall estimates of $_{mol}$FOB presented here may thus be biased. Accurately assessing the effects of this imperfect detectability on parameters estimated from longitudinal genotyping data, such as $_{mol}$FOB, requires complex mathematical modeling (**Bretscher et al., 2010**; **Felger et al., 2012**; **Sama et al., 2005**; **Sama et al., 2006**). However, although clonal detectability has been shown to decrease with age (**Felger et al., 2012**; **Sama et al., 2006**) and MOI (**Koepfli et al., 2011**), it is unlikely to vary substantially within our cohort's age range and transmission setting. Hence the observed differences in $_{mol}$FOB are likely to accurately reflect the relative differences in individual exposure as well as in population transmission levels within the study area.

Evaluating the impact of malaria control efforts requires monitoring changes in malariological metrics over extended periods of time. Drawing comparisons between studies performed at different times in different age groups is particularly challenging because of the interplay of past and current exposure to infective bites and the resulting anti-malarial immunity in the study population of a certain age. In our cohort, fewer *P. vivax* clinical episodes than *P. falciparum* clinical episodes were detected despite a higher incidence of *P. vivax* blood-stage infections, which is consistent with earlier studies in children of similar age (**Michon et al., 2007**). The very low incidence of clinical *P. vivax* episodes in our cohort, at 0.16 clinical episodes/year (placebo arm [**Robinson et al., 2015**]), contrasts drastically with that of 2.46 *P. vivax* clinical episodes/year observed in an earlier observational cohort of younger children aged 1–4 years from the same area (**Lin et al., 2010**). The 3-fold difference in *Pv*-$_{mol}$FOB between the two cohorts seems modest when compared to the 15-fold difference in the incidence of clinical *Pv* episodes (**Koepfli et al., 2013**). This suggests that the much lower incidence of *P. vivax* clinical illness in 5–10 years old children of this study is more likely explained by an advanced state of immunity to *P. vivax* compared to the younger children of the earlier cohort than the drop in *P. vivax* transmission. Consistently, age emerged as the strongest factor associated with protection against *P. vivax* clinical episodes, *Pv*-$_{mol}$FOB and *P. vivax* parasite density. Like in the previous cohort of younger children from neighboring villages (**Lin et al., 2010**) the incidence clinical *P. vivax* clinical episodes dropped significantly with age. Unlike in the previous cohort of younger children (**Koepfli et al., 2013**), in this cohort we additionally observed a drop in *Pv*-$_{mol}$FOB as well as *P. vivax* densities with age (**Table 4** and **Supplementary file 3**). This is a further indication of the substantial clinical immunity to *P. vivax* acquired during years of past exposure in the children of this study, which is still ongoing after the age of five.

In sharp contrast to the age-dependent decline of *P. vivax* clinical episode incidence, no age-dependent decrease in the incidence of clinical episodes was observed for *P. falciparum*. In a previous cohort study conducted in 2004 in 5–14 year old children in an area from PNG with substantially higher transmission levels (mean incidence risk 5.0 versus 0.8 infections/year, **Michon et al., 2007**; **Robinson et al., 2015**), the risk of moderate- to high-density *P. falciparum* infections decreased significantly with age (**Michon et al., 2007**). Clinical immunity to *P. falciparum* in children of this earlier cohort was not only significantly further advanced compared to children of this cohort, but in addition, transmission was more homogeneous in the area of that study. As a consequence age was a much better marker of life-time exposure and thus immune status compared to the present cohort.

In this cohort, exposure to *P. falciparum* infections was highly heterogeneous between study participants. Mathematical modeling suggests that at heterogeneous transmission, changes of parasite prevalence and clinical episode incidence with age are less pronounced compared to settings with homogeneous transmission (**Ross and Smith, 2010**). Although no age trends were observed for *P. falciparum* in this cohort, when children were stratified into groups ranging from low to high exposure we found that the proportion of *P. falciparum* clinical episodes relative to new infections decreased with increasing exposure. This could either reflect the development of clinical immunity in highly exposed children, or premunition, a proposed mechanism by which established infections help to control superinfections by immunological cross-protection (**Sergent and Parrot, 1935**; **Smith et al., 1999**). In settings of decreasing and heterogeneous transmission, age alone may therefore not be a suitable marker of immunity to *P. falciparum*. Instead, combining age and $_{mol}$FOB to estimate cumulative life-time exposure may provide a more accurate surrogate measure of the extent of acquired clinical immunity. With *P. falciparum* transmission declining in PNG due to successful malaria control strategies (**Koepfli et al., 2015**), it is conceivable that immunity against *P. falciparum* will develop more slowly, shifting the burden of disease towards older age groups or towards more complex, non-linear age patterns. This delay in immune acquisition is however more than compensated by the overall much lower incidence of clinical malaria clinical episodes: in cohort studies in children younger than 4 years from Maprik district, clinical *P. falciparum* incidence had dropped from 2.56 clinical episodes/year before (observational cohort, [**Lin et al., 2010**; **Mueller et al., 2012**]) to 0.67 clinical episodes/year immediately after the free LLIN distribution campaign (placebo arm, [**Betuela et al., 2012**]).

Finally, given that four *Plasmodium* species co-exist in PNG, there has long been considerable interest in potential mechanisms of cross-species immunity and mixed species interactions (**Mueller et al., 2009a**; **Bruce et al., 2000**; **Smith et al., 2001**; **Mehlotra et al., 2000**). However, there is so far no consistent evidence for the presence or absence of cross-protection among *Plasmodium* species. *P. vivax* and *P. falciparum* infections in our study were concentrated in the same children and villages (**Figure 3**), likely due to overlapping focal transmission for *P. falciparum* and *P. vivax* and thus potentially high co-infection rates in mosquitoes. Contrary to an earlier cohort in younger PNG children that found a decreased risk of *P. falciparum* clinical episodes after PQ radical cure (**Betuela et al., 2012**), we found indications for an increased risk of *P. falciparum* illness after clearance of *P. vivax* hypnozoites using PQ. Our data suggests that in individuals with substantial clinical immunity against *P. vivax,* a concurrent *P. vivax* infection may provide protection against *P. falciparum* clinical episodes by limiting *P. falciparum* densities (**Table 6**, **Supplementary file 3**). However, the comparably small number of clinical episodes in this and the earlier contrasting study does not allow an in-depth analysis of causal relationships and therefore does not allow firm conclusions on the potential effects and mechanisms of cross-species interactions in mixed infections.

In conclusion, this study provides detailed insight into the changing epidemiology of malaria in PNG children under sustained malaria control, by using $_{mol}$FOB as a powerful measure to quantitatively investigate patterns of new mosquito-derived *P. falciparum* and *P. vivax* infections versus those for *P. vivax* relapsing infections, as well as spatial and age trends in exposure to these infections. Striking heterogeneity in malaria transmission between villages as well as in individual exposure to new *P. falciparum* and *P. vivax* infections persisted in our study area despite very high use of LLINs. This presents a significant challenge for on-going malaria control efforts. The comparable patterns of new mosquito-derived *P. falciparum* and *P. vivax* infections indicate that sustained use of LLINs does result in a comparable reduction in transmission of both species. The higher incidence and prevalence of *P. vivax* infections observed in our data is thus directly linked to its ability to cause relapsing infections, highlighting the crucial role of hypnozoites for *P. vivax* epidemiology and the need to effectively intervene against these hidden stages. Together, these insights provide a crucial link to evaluate the level of *P. vivax* mosquito-based transmission against that of *P. falciparum* and serve to calibrate other standard malaria indicators such as parasite prevalence or incidence of clinical episodes and to ultimately inform new approaches to surveillance and response systems.

Materials and methods

Study design and participants

This study was conducted in six villages in the Albinama and Balif areas, Maprik district, East Sepik Province, PNG between August 2009 and May 2010. The area is serviced by the Albinama health sub-center, Balif aid post and a network of health workers in all study villages. The study design has been described in detail elsewhere (**Robinson et al., 2015**). Briefly, 524 children aged 5–10 years whose parents provided written informed consent for their participation were enrolled and randomized to receive either chloroquine (CQ, days 1–3, total dose 25 mg/kg), artemeter-lumefantrine (Coartem, AL, days 11–13, 2 mg/kg A, 12 mg/kg L) and primaquine (PQ, days 1–20, 0.5 mg/kg/day); or CQ (days 1–3), AL (days 11–13), and placebo (days 1–20) over 20 days of directly observed treatment (DOT_{1-20}) in a double-blinded manner. Children were actively visited and examined for signs and symptoms of malaria fortnightly at their schools for 8 months. In addition, passive surveillance was provided by the local health centre, aid post and village health workers throughout the study period. Finger-prick blood samples (250 µl) were collected at fortnightly active-follow-up visits in the first 12 weeks and monthly thereafter, as well as from symptomatic children detected during active or passive morbidity surveillance. Symptomatic children were tested for malaria infection with rapid diagnostic test (RDT, CareStartMalaria pLDH/HRP2 Combo, AccessBio, USA), and only RDT and or LM-confirmed *Plasmodium* infections of any density were treated with a 3 day course of AL.

Household, village and health facility location data was collected using a handheld GPS receiver (Garmin GPSmap62sc) and maps were prepared using ArcGIS 10.2 (Esri, USA).

The study received ethical clearance from the PNG IMR Institutional Review Board (0908), the PNG Medical Advisory Committee (09.11), the Ethics Committee of Basel 237/11 and was conducted in full concordance with the Declaration of Helsinki. The study was registered on ClinicalTrials.gov (NCT02143934).

Laboratory methods

All blood samples were examined by LM and qPCR for detection and speciation of *Plasmodium* infections as described earlier (**Robinson et al., 2015**). Each blood slide was read independently by two skilled microscopists and re-read by an expert microscopist in case of discrepancies in positivity, speciation or density ($\geq$2 x $\log_{10}$ difference). Thick blood films were examined by LM for 200 fields (1000x magnification) before being declared parasite-negative. Parasite density was converted from the number of parasites per 200–500 white blood cells (WBC) to parasites/µl assuming 8000 WBC/µl (WHO malaria microscopy training guide) and calculated as the geometric mean of all positive reads.

DNA was extracted from the red blood cell pellet using the FavorPrep 96-well genomic DNA extraction kit (Favorgen). Samples carrying any *Plasmodium spp.* infection were identified using a generic qPCR (**Wampfler et al., 2013**) and positives were subsequently tested in species-specific qPCRs (**Rosanas-Urgell et al., 2010**; **Wampfler et al., 2013**). All qPCRs targeted the *small subunit (18S) ribosomal RNA* gene and were performed as simplex (*P. vivax* and *P. falciparum*) or duplex qPCR (*P. malariae*, *P. ovale*). The concentration of target copies per µl of DNA was determined relative to a dilution row of standard plasmid as previously described (**Rosanas-Urgell et al., 2010**). The qPCR limit of detection (LOD) was determined using a standard plasmid dilution row and defined as the last point with more than 50% of replicates positive. The LOD was 2 target copies/µl DNA, equaling 4 target copies/reaction, for all qPCRs. All samples that crossed the fluorescence threshold were scored as positive for species-specific qPCRs. In all samples positive in *P. falciparum* and/or *P. vivax* qPCRs, individual parasite clones were distinguished by genotyping the length-polymorphic *Pf-msp2* or *Pv-msp1F3* marker genes using capillary electrophoresis for highly precise fragment sizing (**Koepfli et al., 2013**; **Koepfli et al., 2011**; **Falk et al., 2006**; **Schoepflin et al., 2009**). MOI was determined by counting the number of detected *Pf-msp2* or *Pv-msp1F3* alleles per sample. $_{mol}$FOB was calculated from the number of new parasite clones detected per child or per sampling interval in the peripheral blood, divided by the individual time at risk or length of the interval. A new infection was defined as a *Pf-msp2* or *Pv-msp1F3* allele not present in the two preceding genotyping-positive samples collected during active or passive surveillance (**Figure 1—figure supplement 1**). Imperfect diagnostic detectability was not further adjusted for.

Statistical analysis

Children were considered at risk for clinical malaria clinical episodes until the end of the study or until they were censored (on the last visit before two consecutively missed scheduled follow-up visits [*Robinson et al., 2015*]). For clinical endpoints, time-at-risk (TAR) was not further adjusted for interim missed follow-up visits because the intense active and passive case detection presumably led to detection of all malaria clinical episodes. In contrast, TAR for analysis of molecular data (e.g., $_{mol}$FOB) was reduced by the duration of the missed interval if a child was not seen by the study team for six weeks or more (≥42 days). Children with a TAR of less than 3 months (<84 days) were excluded. This resulted in an analyzed population of 466 children (characterized in *Table 1*) of which 430 (92.3%) completed the whole follow-up period, with a median of 15 (IQR: 13–17) study contacts and mean TAR of 186 days (IQR 168–223 days).

Time to first *Plasmodium* infection by qPCR and LM and its association with covariates were modeled using Cox regression, and the proportional hazards assumption was checked using the test based on the Schoenfeld residuals. Multiple failure Cox regression was used to model the time to *P. vivax* and P. *falciparum* clinical episodes.

For statistical analysis, a malaria clinical episode was defined as fever (>37.5°C axillary) plus the presence of LM-detectable parasites, irrespective of RDT result or antimalarial treatment during the field visit. Negative binomial generalized estimating equations (GEE) with log link function using an exchangeable correlation structure were used to model incidence of new infections with *P. falciparum* and *P. vivax* per sampling interval. For these analyses, the time at risk was restricted to the intervals where $_{mol}$FOB could be estimated (i.e., starting in the third follow-up interval). A binomial GEE with logit link function using an exchangeable correlation structure was used to model the odds of a *P. falciparum* clinical episode per interval. Gaussian GEEs with log link function using an exchangeable correlation matrix were used to model log-transformed qPCR parasite densities in qPCR-positive samples, measured as 18S rRNA copy numbers/µl blood. In the GEE and Cox models where $_{mol}$FOB was a covariate, it was included as a time-varying covariate. When modelling $_{mol}$FOB (*Table 4*) and the odds of clinical episodes (*Table 6*) using GEEs, $_{mol}$FOB was calculated for each follow-up interval and used as predictor. In Cox models investigating the risk of clinical episodes (*Table 5*), $_{mol}$FOB was calculated based on the new infections up to the time of failure and used as predictor. In exploratory preliminary analyses we tested for a wide variety of interactions between covariates including interactions between all combinations of $_{mol}$FOB, enrolment infection status, age, village and bednet-usage. All analyses were done using STATA v14 and R.

Maps were drawn using Arcgis 10.1 (Esri Inc.). Ordinary kriging was used to generate the contour maps. Semivariograms were used as the mathematical forms used to express autocorrelation. Input variables for the spatial models were (i)$_{mol}$FOB (prediction of independent variable, $_{mol}$FOB) using the model presented in *Table 4* (negative binomial GEE) for *Pf* and *Supplementary file 4* for *Pv* (same as that shown in *Table 4* but with primaquine and placebo arms combined), resulting in *Figure 3* Panels A and B; (ii) relative risk of clinical episodes as predicted by the model shown in *Table 5*, resulting in Panels C and D of *Figure 3*. Relative standard error maps were generated by dividing the absolute standard error map by the model prediction map.

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Author contributions

Natalie E Hofmann, Data curation, Formal analysis, Investigation, Methodology, Writing—original draft; Stephan Karl, Formal analysis, Writing—original draft; Rahel Wampfler, Investigation, Methodology, Writing—review and editing; Benson Kiniboro, Albina Teliki, Data curation, Project administration, Writing—review and editing; Jonah Iga, Andreea Waltmann, Data curation, Methodology, Writing—review and editing; Inoni Betuela, Supervision, Investigation, Project administration, Writing—review and editing; Ingrid Felger, Conceptualization, Supervision, Investigation, Methodology, Writing—review and editing; Leanne J Robinson, Conceptualization, Data curation, Formal analysis, Supervision, Investigation, Writing—original draft, Project administration, Writing—review and editing; Ivo Mueller, Conceptualization, Formal analysis, Supervision, Funding acquisition, Investigation, Writing—review and editing

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Ethics

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Additional files

Supplementary files

- Supplementary file 1. Univariate factors. Table 1. Univariate/PQ-adjusted predictors for time to recurrent blood-stage infection with *Plasmodium* species by qPCR. Table 2. Univariate (*P. vivax*) and univariate/PQ-treatment-adjusted (*P. falciparum*) predictors for *Pv*- and $Pf\text{-}_{mol}$FOB by follow-up interval. Table 3. Univariate/PQ-treatment-adjusted predictors for time to *P. vivax* and *P. falciparum* episodes. Table 4. Univariate/PQ-treatment-adjusted predictors for odds of *P. falciparum* clinical episodes.

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- Supplementary file 2. Multivariable predictors for time to recurrent blood-stage infection with *Plasmodium* species by LM.

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- Supplementary file 3. Multivariable predictors for *P. falciparum* and *P. vivax* density by qPCR during follow-up.

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- Supplementary file 4. Multivariable predictors of $Pv\text{-}_{mol}$FOB (combining primaquine and placebo arms) per follow-up interval. This model is similar to that presented in ***Table 4*** in the main text but combines the treatment arms for *P. vivax*. Model predictions from this model were used for mapping molFOI in ***Figure 3B***.

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Major datasets

The following dataset was generated:

Author(s)	Year	Dataset title	Dataset URL	Database, license, and accessibility information
Natalie E Hofmann, Stephan Karl, Rahel Wampfler, Benson Kiniboro, Albina Teliki, Jonah Iga, Andreea Waltmann, Inoni Betuela, Ingrid Felger, Leanne J Robinson, Ivo Mueller	2017	Data from: The complex relationship of exposure to new Plasmodium infections and incidence of clinical malaria in Papua New Guinea	http://dx.doi.org/10.5061/dryad.f9154	Available at Dryad Digital Repository under a CC0 Public Domain Dedication

The following previously published dataset was used:

Author(s)	Year	Dataset title	Dataset URL	Database, license, and accessibility information
Robinson LJ, Wampfler R, Betuela I, Karl S, White MT, Li Wai Suen CSN, Hofmann NE, Kiniboro B, Waltmann A, Brewster J, Lorry L, Tarongka N, Samol L, Silkey M, Bassat Q, Siba PM, Schofield L, Felger I, Mueller I	2015	Data from: Strategies for understanding and reducing the Plasmodium vivax and Plasmodium ovale hypnozoite reservoir in Papua New Guinean children: a randomised placebo-controlled trial and mathematical model	http://dx.doi.org/10.5061/dryad.m1n03	Available at Dryad Digital Repository under a CC0 Public Domain Dedication

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Betuela I, Rosanas-Urgell A, Kiniboro B, Stanisic DI, Samol L, de Lazzari E, Del Portillo HA, Siba P, Alonso PL, Bassat Q, Mueller I. 2012. Relapses contribute significantly to the risk of plasmodium vivax infection and disease in papua new guinean children 1-5 years of age. *The Journal of Infectious Diseases* **206**:1771–1780. DOI: https://doi.org/10.1093/infdis/jis580, PMID: 22966124

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SUPPLEMENT

Figure 1 – figure supplement

Figure 1 – figure supplement 1: Definition of new infections for calculating $_{mol}$FOB. Definition of *P. falciparum* new infections in two exemplary children is shown. The study design and timelines of follow-up are shown in upper panel: enrolment visit ("E"), followed by radical treatment (black bar "T") and 235 days of follow-up. The presence of *P. falciparum* clones by sampling visit is visualized below. Columns represent sampling visits, rows represent *P. falciparum msp2* alleles, i.e. distinct *P. falciparum* clones. Grey solid circles, *P. falciparum* negative sample, grey open circle, missing sample due to missed follow-up visit; red circle: sample positive for respective *Pf-msp2* allele. New infections were defined as a positive sample preceded by two samples negative for this allele (black rectangles), excluding missed samples (see Child 2, allele F, days 120-200). The time point of new infections is marked by arrows for the two children.

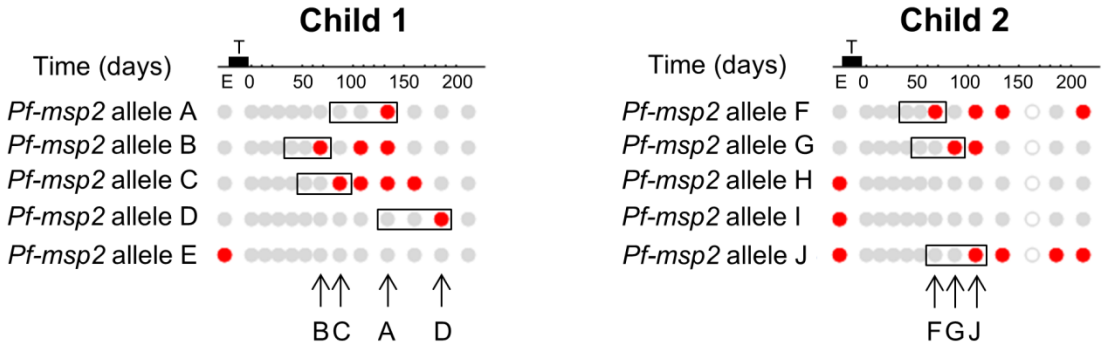
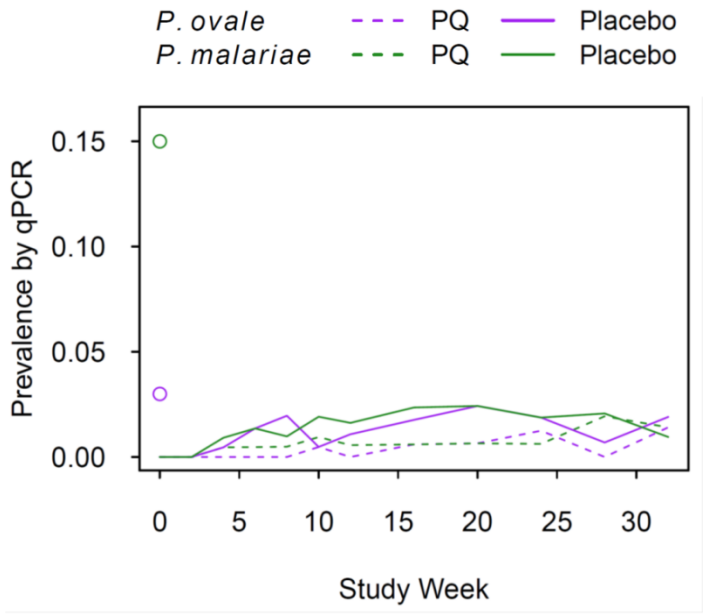


Figure 1 – figure supplement 2: *P. ovale* and *P. malariae* prevalence by qPCR during follow-up. Purple lines, *P. ovale*; green lines, *P. malariae*; solid lines, Placebo arm; dashed lines, PQ arm. Open circles mark enrolment qPCR prevalence for each species.



Supplementary File 1 – Univariate factors

Table 1. Univariate/PQ-adjusted predictors for time to recurrent blood-stage infection with *Plasmodium* species by qPCR.

Variable	*P. vivax*			*P. falciparum*			*P. malariae*			*P. ovale*		
	HR[1]	CI_{95}	*p*-value	HR[1]	CI_{95}	*p*-value	HR[1]	CI_{95}	*p*-value	HR[1]	CI_{95}	*p*-value
PQ treatment	0.27	0.20-0.36	<0.001	0.79	0.57-1.09	0.057	0.60	0.26-1.38	0.233	0.40	0.17-0.96	0.040
Age	0.94	0.86-1.02	0.155	0.97	0.87-1.08	0.542	0.95	0.73-1.23	0.684	0.90	0.69-1.18	0.447
LLIN use at enrolment	0.38	0.25-0.58	<0.001	0.44	0.27-0.72	0.001	0.77	0.18-3.28	0.724	0.51	0.15-1.70	0.271
Hb at enrolment (g/dl)	0.81	0.73-0.89	<0.001	0.76	0.68-0.86	<0.001	0.66	0.50-0.87	0.003	0.78	0.58-1.04	0.087
Village												
Albinama (ref)	1			1			1			1		
Amahup	0.44	0.28-0.69	<0.001	0.55	0.29-1.05	0.068	0.32	0.06-1.67	0.178	2.57	0.27-24.73	0.414
Balanga	2.23	1.46-3.39	<0.001	2.06	1.15-3.69	0.016	1.34	0.35-5.12	0.665	7.28	0.81-65.11	0.076
Balif	0.96	0.63-1.50	0.843	0.57	0.29-1.12	0.101	0.21	0.02-1.79	0.153	4.13	0.46-36.92	0.205
Bolumita	4.92	3.29-7.36	<0.001	6.79	4.15-11.12	<0.001	3.08	1.04-9.11	0.041	22.40	2.88-174.39	0.003
Numangu	0.91	0.48-1.71	0.765	2.43	1.25-4.73	0.009	1.36	0.26-7.04	0.711	3.60	0.22-57.66	0.365
Infection status at enrolment (by qPCR)												
Uninfected (ref)	1			1			1			1		
P. vivax	1.34	0.97-1.87	0.079	1.49	0.94-2.37	0.090	1.03	0.23-4.62	0.971	1.97	0.63-6.23	0.246
P. falciparum	2.52	1.64-3.89	<0.001	3.11	1.77-5.46	<0.001	6.09	1.63-22.82	0.007	2.89	0.69-12.12	0.146
P. malariae	1.37	0.63-2.98	0.426	1.74	0.68-4.45	0.250	9.81	2.17-44.38	0.003	2.63	0.31-22.63	0.378
Mixed *P.f. or P.v.*[2]	3.39	2.37-4.87	<0.001	5.84	3.75-9.10	<0.001	6.64	2.03-21.72	0.002	5.50	1.79-16.88	0.003

[1] PQ treatment, univariate HR. All other HRs are adjusted only for PQ treatment. HRs were modeled using Cox proportional hazard regression.

[2] Mixed infection including *P. falciparum* or *P. vivax* infection in conjunction with one or more other *Plasmodium spp.*

Table 2. Univariate (*P. vivax*) and univariate/PQ-treatment-adjusted (*P. falciparum*) predictors for *Pv*- and *Pf*-$_{mol}$FOB by follow-up interval.

Variable	*P. vivax*						*P. falciparum*		
	PQ arm			placebo arm			Combined arms		
	IRR[1]	CI_{95}	*p*-value	IRR[1]	CI_{95}	*p*-value	IRR[2]	CI_{95}	*p*-value
PQ treatment	n.a.[3]	n.a.	n.a	n.a.	n.a.	n.a.	0.94	0.64-1.39	0.785
New *P. falc.* infections in interval	1.78	1.18-2.69	0.006	1.52	1.11-2.10	0.010	n.a.	n.a	n.a
New *P. vivax* infections in interval	n.a.	n.a	n.a.	n.a.	n.a.	n.a.	1.41	1.13-1.75	0.002
Age	0.79	0.67-0.93	0.004	0.94	0.85-1.04	0.23	0.94	0.82-1.18	0.39
LLIN at enrol.	0.58	0.32-1.02	0.060	0.50	0.34-0.74	<0.001	0.53	0.31-0.91	0.022
Haemoglobin at enrol. (g/dl)	0.90	0.79-1.01	0.081	0.94	0.86-1.03	0.20	0.84	0.77-0.91	<0.001
Village									
Albinama (ref)	-	-		-	-		-	-	
Amahup	0.02	0.00-0.12	<0.001	0.50	0.30-0.83	0.007	0.52	0.2-1.06	0.072
Balif	0.93	0.41-2.11	0.874	1.66	1.09-2.52	0.019	1.96	1.06-3.64	0.032
Balanga	0.25	0.09-0.74	0.013	1.00	0.64-1.56	1.000	0.74	0.36-1.52	0.416
Bolumita	1.98	1.05-3.78	0.036	3.18	2.19-4.59	<0.001	7.80	4.48-13.55	<0.001
Numangu	0.46	0.14-1.54	0.213	0.75	0.40-1.44	0.391	2.85	1.45-5.62	0.002
Study Day									
Day 0-35 (ref)	-	-					-	-	
Day 36-80	1.46	0.60-3.55	0.410	1.90	1.21-2.75	0.001	2.54	1.45-4.45	0.001
Day 81-175	1.35	0.61-2.99	0.450	0.81	0.5-1.19	0.285	1.12	0.67-1.88	0.655
Day >175	0.66	0.26-1.67	0.480	0.56	0.37-0.84	0.005	0.91	0.49-1.71	0.772

[1] *P. vivax*, stratified by treatment arm. Univariate IRRs were modeled per sampling interval using negative binomial generalized estimating equations allowing for repeated visits with log-link and an exchangeable correlation structure.

[2] *P. falciparum*, combined treatment arms. PQ treatment, univariate IRR. All other IRRs are adjusted only for PQ treatment. IRRs were modeled per sampling interval using negative binomial generalized estimating equations allowing for repeated visits with log-link and an exchangeable correlation structure.

[3] n.a., not applicable.

Table 3. Univariate/PQ-treatment-adjusted predictors for time to *P. vivax* and *P. falciparum* episodes.

Variable	*P. vivax*			*P. falciparum*		
	HR[1]	**CI_{95}**	***p*-value**	**HR[1]**	**CI_{95}**	***p*-value**
PQ treatment	0.50	0.21-1.56	0.11	1.72	1.01-2.93	0.04
P. vivax $_{mol}$FOB[3]	1.07	1.05-1.09	<0.001	n.a.	n.a.	n.a.
P. falciparum $_{mol}$FOB[3]	n.a.	n.a.	n.a.	1.18	1.15-1.21	<0.001
Age	0.66	0.50-0.87	0.003	0.95	0.80-1.11	0.50
LLIN at enrolment	0.88	0.21-3.60	0.86	0.45	0.22-0.96	0.038
Hb at enrolment (g/dl)	0.76	0.58-0.99	0.045	0.76	0.63-0.93	0.006
Village						
Albinama (ref)	-	-	-	1	-	-
Amahup	0.72	0.18-2.78	0.637	0.54	0.15-1.94	0.346
Balif	1.50	0.45-5.02	0.506	0.96	0.29-3.19	0.949
Balanga	0.68	0.16-3.02	0.617	1.25	0.47-3.36	0.656
Bolumita	2.12	0.69-6.53	0.189	3.78	1.61-8.90	0.002
Numangu	1.13	0.23-5.69	0.876	5.70	2.43-13.30	0.001
Infection status at enrolment (by qPCR)						
Uninfected (ref)	-	-	-	-	-	-
P. vivax	2.41	0.94-6.18	0.068	3.21	1.67-6.16	<0.001
P. falciparum	0.88	0.33-2.36	0.806	1.41	0.72-2.78	0.319
Mixed *P.f.* or *P.v.*	1.38	0.39-4.95	0.620	1.43	0.48-4.27	0.517

[1] PQ treatment, univariate HR. All other HRs are adjusted only for PQ treatment. AHRs were modeled using multiple failure Cox proportional hazard regression.

[2] n.a., not applicable

[3] Average $_{mol}$FOB until the time of failure (time-varying covariate).

Table 4. Univariate/PQ-treatment-adjusted predictors for odds of *P. falciparum* clinical episodes.

	***P. falciparum* episode**		
Variable	**OR[1]**	**CI_{95}**	***p*-value**
PQ treatment	1.63	0.94-2.83	0.08
P. vivax qPCR positive[2]	0.70	0.34-1.45	0.35
P. falciparum $_{mol}$FOB	1.22	1.14-1.31	<0.001
Age	0.89	0.76-1.06	0.20
LLIN at enrolment	0.39	0.18-0.86	0.02
Haemoglobin (g/dl)	0.97	0.85-1.11	0.68
Village			
Albinama (ref)	-	-	
Amahup	0.43	0.12-1.55	0.198
Balif	0.94	0.27-3.31	0.925
Balanga	1.34	0.46-3.85	0.592
Bolumita	4.14	1.59-10.74	0.004
Numangu	5.90	2.34-14.89	<0.001
Study Day			
Day 0-80 (ref)	-	-	
Day 81-175	0.87	0.47-1.61	0.649
Day >175	0.73	0.36-1.47	0.376

[1] PQ treatment, univariate OR. All other ORs are adjusted only for PQ treatment. ORs were modeled using a binomial generalized estimating equation with logit link function using an exchangeable correlation structure.
[2] Determined as *P. vivax* positive at the same or previous sampling visit.

Supplementary File 2 - Multivariable predictors for time to recurrent blood-stage infection with *Plasmodium* species by LM.

Variable	*P. vivax*			*P. falciparum*		
	AHR[1]	CI_{95}	*p*-value	AHR[1]	CI_{95}	*p*-value
PQ treatment	0.17	0.11-0.24	<0.001	0.76	0.50-1.17	0.216
Age	0.85	0.77-0.95	0.004	1.16	1.01-1.33	0.037
LLIN use at enrolment	0.76	0.45-1.30	0.319	0.56	0.31-1.02	0.056
Hb at enrolment (g/dl)	0.86	0.76-0.97	0.18	0.78	0.67-0.91	0.002
Village						
Albinama (ref)	1			1		
Amahup	0.35	0.19-0.63	0.001	1.19	0.44-3.20	0.736
Balanga	1.49	0.88-2.51	0.139	3.17	1.24-8.12	0.016
Balif	1.12	0.69-1.84	0.644	1.25	0.45-3.46	0.673
Bolumita	3.38	1.97-5.82	<0.001	9.07	3.76-21.87	<0.001
Numangu	0.59	0.25-1.35	0.208	5.93	2.27-15.50	<0.001
Infection status at enrolment (by qPCR)						
Uninfected (ref)	1			1		
P. vivax	1.49	0.98-2.25	0.062	1.21	0.63-2.35	0.562
P. falciparum	1.10	0.61-1.99	0.747	1.53	0.74-3.15	0.251
P. malariae	1.28	0.53-3.11	0.575	0.87	0.25-2.99	0.828
Mixed *P.f. or P.v.*[2]	1.94	1.18-3.21	0.009	2.24	1.18-4.23	0.013

[1] AHRs were modeled using Cox proportional hazard regression.

[2] Mixed infection including *P. falciparum* or *P. vivax* infection in conjunction with one or more other *Plasmodium spp.*

Supplementary file 3 - Multivariable predictors for *P. falciparum* and *P. vivax* density by qPCR during follow-up.

	***P. vivax* density[1] by qPCR**			***P. falciparum* density[1] by qPCR**		
Variable	**exp(β)[2]**	**CI_{95}**	***p*-value**	**exp(β)[2]**	**CI_{95}**	***p*-value**
PQ treatment	1.16	0.85-1.58	0.354	0.66	0.39-1.10	0.112
Fever (>37.5°C axillary)	0.99	0.57-1.71	0.960	7.38	3.60-15.14	<0.001
P. falc. $_{mol}$FOB	n.a.	n.a.	n.a.	1.07	1.00-1.15	0.039
P. *vivax* qPCR positive	n.a.	n.a.	n.a.	0.45	0.25-0.81	0.008
P. vivax $_{mol}$FOB	1.03	1.01-1.05	0.006	n.a.	n.a.	n.a.
P. *falc.* qPCR positive	1.11	0.81-1.52	0.529	n.a.	n.a.	n.a.
Age	0.90	0.83-0.98	0.016	0.96	0.80-1.14	0.645
LLIN at enrol.	1.03	0.71-1.49	0.883	0.41	0.15-1.08	0.072
Hb at enrolment (g/dl)	1.11	1.00-1.22	0.050	0.91	0.74-1.11	0.353
Village						
Albinama	1			1		
Amahup	1.02	0.56-1.86	0.941	0.83	0.21-3.36	0.796
Balif	0.99	0.71-1.37	0.933	1.26	0.38-4.14	0.707
Balanga	0.71	0.49-1.03	0.071	3.35	0.78-14.43	0.104
Bolumita	0.79	0.57-1.10	0.168	0.76	0.26-2.27	0.629
Numangu	0.93	0.48-1.81	0.833	0.82	0.23-2.91	0.757
Month of follow-up	0.77	0.73-0.82	<0.001	0.82	0.71-0.94	0.004

[1] Parasite densities by qPCR were assessed as *18S rRNA* copy numbers/µl blood and log_{10} transformed.

[2] Estimates were obtained using gaussian generalized estimating equations with logit-link allowing for repeated visits.

[3] n.a., not applicable

Supplementary file 4 - Multivariable predictors of *Pv*-$_{mol}$FOB (combining primaquine and placebo arms) per follow-up interval. This model is similar to that presented in Table 4 in the main text but combines the treatment arms for *P. vivax*. Model predictions from this model were used for mapping molFOI in Figure 3B.

Variable	*P. vivax (combined)*		
	IRR[1]	**CI$_{95}$**	***p*-value**
PQ treatment	0.27	0.2-0.36	<0.001
New *P. falc.* infections in interval[2]	1.18	0.96-1.45	0.113
Age	0.92	0.85-1	0.043
LLIN at enrolment	0.74	0.54-1.01	0.057
Hb at enrolment (g/dL)	0.9	0.84-0.97	0.007
Village			
Albinama (ref)	1		
Amahup	0.34	0.21-0.56	<0.001
Balif	1.39	0.95-2.03	0.087
Balanga	0.78	0.51-1.18	0.233
Bolumita	2.22	1.52-3.23	<0.001
Numangu	0.64	0.35-1.16	0.138
Study Day			
Day 0-35 (ref)	1		
Day 36-80	1.81	1.28-2.57	0.001
Day 81-175	1.01	0.71-1.44	0.945
Day >175	0.58	0.4-0.85	0.005

[1]IRRs were modeled per sampling interval using negative binomial generalized estimating equations allowing for repeated visits with log-link and an exchangeable correlation structure.

[2] $_{mol}$FOB in the follow-up interval (time-varying covariate).

Chapter 4:

Dynamics of natural *P. falciparum* infections in PNG

Duration of natural *Plasmodium falciparum* infections in Papua New Guinea

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Abstract

BACKGROUND: The duration of natural *P. falciparum* infections is a fundamental parameter of the parasite's infection dynamics. It is inherently difficult to study in endemic settings because of frequent super-infection and imperfect detectability of parasite clones. The effects of age or exposure to new infections on the duration of *P. falciparum* infections are poorly studied. In non-African settings, co-infection with other *Plasmodium* species may further influence the dynamics of *P. falciparum* infections.

METHODS: We estimated *P. falciparum* infection duration and the force of infection (FOI), i.e. the acquisition rate of new infections, using the triplet model that allows for imperfect detectability of clones. Samples were obtained from two treatment-to-reinfection cohorts in settings of different transmission intensity in Papua New Guinea (PNG). Both studies comprised initial clearance of blood-stage parasites followed by six to eight months of two- to four-weekly blood sampling. *P. falciparum* clones were distinguished by *msp2* genotyping.

RESULTS: *P. falciparum* infection durations in PNG were generally short, and lasted 38 days (median, CI_{95}: 34-42) in the high-transmission setting and 62 days (median, CI_{95}: 42-127) in the intermediate-transmission setting. In both studies infection durations were longer in the higher exposed children. No pronounced trend in infection duration with age was observed. Clone detectability was higher in the high-transmission setting (median 78%, CI_{95}: 72-82), despite the higher number of concurrent clones compared to the intermediate-transmission setting. Clone detectability at intermediate transmission varied with exposure and age and ranged between 49-73%. In the high-transmission setting, the duration of individual infections almost matched the length of sampling intervals. Here, adjusting not only for clone detectability but also infection duration substantially increased the FOI estimate.

CONCLUSIONS: *P. falciparum* infections in PNG children are shorter than those in similarly aged Ghanaian children. This may reflect evolutionary adaptation of the parasite to settings of differing seasonality, differences in *Plasmodium* species composition, or differences in parasite diversity encountered by the host's immune system.

Introduction

Asymptomatic, often chronic infections make up the majority of malaria infections and are considered the main reservoir sustaining malaria transmission (1), yet surprisingly few studies have investigated a fundamental parameter of infection dynamics: the duration of a distinct parasite infection in a host (2–5). The persistence of an individual infection can only be monitored by genotyping this parasite clone as frequent super-infections prohibit determining infection duration by microscopy or diagnostic PCR. Unlike *P. vivax*, *P. falciparum* has no dormant liver stage that could re-activate after clearance of a primary infection. Therefore prolonging the duration of an infection is a pivotal strategy to maximize transmission success for *P. falciparum*. To permit persistence in the host despite immunological recognition, *P. falciparum* switches the major parasite antigen displayed on the infected erythrocyte surface by mutually exclusive expression of highly diverse *var*-genes (6). The duration of an untreated *P. falciparum* infection in the human host thus depends on the host's anti-malarial acquired immunity counterbalanced by the parasite's ability to evade this immune response (7).

Some of the most detailed data on the course and duration of *P. falciparum* infections derive from treatment of neurosyphilis patients after deliberate malaria infection in the pre-antibiotic era (8–10). Parasitemia lasted on average 211 days and rarely exceeded one year (8,10,11). However, the relevance of this data for natural *P. falciparum* infections in endemic settings is questionable, as hosts were usually malaria-naïve, received one highly controlled infection and were often treated with sub-curative treatment doses. In contrast, people living in endemic countries are frequently super-infected, acquire semi-immunity to malaria with repeated exposure to diverse parasites (12) and only seek treatment following symptoms. Naturally clearing malaria infections are thus rarely detected in endemic settings and their dynamics are challenging to study.

Differentiation of parasite clones by genotyping is required for studying individual malaria infections in endemic settings where super-infections are common. However, several factors can impede the detection of parasite clones in peripheral blood samples: (i), technical problems associated with genotyping PCR limit the capability to amplify minority clones (Messerli & Hofmann *et al.*, submitted); (ii), biological features of *P. falciparum* sometimes prevent detection of a parasite clone, e.g. when sequestration of late-stage parasites in deep organs leads to periodical absence of synchronized clones from the peripheral circulation; (iii), fluctuating parasite densities may occasionally fall below the limit of detection of PCR; or (iv), the small volume sampled may simply not contain a parasite of the minority clone if parasite densities are low. Detectability of *P. falciparum* clones is hence imperfect especially in semi-immune individuals who are able to control parasite densities (13). In a longitudinal cohort in Northern Ghana detectability decreased from 60% in young children to less than 20% in individuals >60 years of age (4). The declining detectability mirrored the decrease of parasite densities with age (4,14). Adjusting for imperfect detectability using mathematical

models is thus crucial to obtain accurate estimates of acquisition or clearance rates of natural *P. falciparum* infections (3,4,15,16).

The effect of natural acquired immunity on acquisition or clearance rates themselves is not well understood. In absence of a direct and individual measure of anti-malarial immunity, age is often utilized as surrogate marker. However, the study of age trends in infection duration requires intensive monitoring of asymptomatic carriers of all ages over extended periods of time, in combination with in-depth molecular analysis of samples, and is thus rarely done. One suitable dataset exists, in which *P. falciparum* high-resolution genotyping was used in a cohort in Northern Ghana covering the whole age range (17). To study the effect of acquired immunity on *P. falciparum* infection duration, this data has been analyzed with more and more complex models (15,3,14,4). Using the "triplet" model approach, average infection duration over all ages was estimated at 168 days. Age-trends in infection duration were non-monotonic with longest infections observed in children aged 5-9 years (216 days), suggesting that no simple linear relation of acquired immunity and *P. falciparum* infection duration exists (4).

Data on the durations of non-African *P. falciparum* infections is scarce. Outside of Africa, *P. falciparum* often co-exists with other malaria species, which may interact and alter the course of an infection (18,19). The few studies assessing *P. falciparum* infection durations in Puerto Rico (20), West Papua (2) or Papua New Guinea (21) are based on blood-slide positivity, and only one dataset comprises molecular analysis of clonal infections with simple adjustment for imperfect detectability (22).

Durations of natural infections are much needed parameters to inform models of malaria transmission. It is crucial to investigate this parameter in different endemic settings, and importantly in areas with different seasonal patterns. To fill this gap in knowledge, the dynamics of natural *P. falciparum* infections were investigated in two cohorts of children from Papua New Guinea (PNG), for which detailed clinical as well as molecular parasitological data was collected (23,24). Detectability-adjusted infection duration and clone acquisition rates (i.e. the force of infection, FOI) were determined using the triplet model. To explore the effect of frequent super-infection on *P. falciparum* infection durations, children were stratified by individual exposure to *P. falciparum* (25) (chapter 3). Individual exposure, besides age, can further serve as proxy for acquired immunity particularly in settings of heterogeneous transmission where age may not be a suitable surrogate marker (chapter 3).

Methods

Field studies

Samples derived from two cohort studies conducted in areas of medium to high perennial transmission along the North Coast of PNG (23,24). The study design was comparable in that both cohorts comprised an initial anti-malarial treatment and regular active as well as close passive follow-up. Details of treatment regimens and study design can be found elsewhere (23,24). Briefly, the first cohort comprised 206 children aged 5-14 years from Mugil area/Madang province who were treated with artesunate monotherapy in June 2004 and subsequently followed for 6 months. Finger-prick blood samples were collected in monthly intervals. The second cohort was performed in Maprik area/East Sepik province, and comprised 504 children aged 5-10 years who received either blood-stage or blood-plus-liver-stage radical cure in August to September 2009 and were followed for 8 months. Finger-prick blood samples were collected in 2-weekly intervals in the first three months of follow-up and monthly thereafter. The two cohorts are further referred to as the "Mugil" and "Maprik" cohorts. In both studies, finger-prick blood samples were collected from children that presented to the collaborating health centers or field team showing signs and symptoms of malaria. Scientific approval and ethical clearance was obtained from the PNG Medical Research Advisory Council, plus the Walter and Eliza Hall Institute and the Veteran's Affairs Medical Center, Cleveland, Ohio, the PNG IMR Institutional Review Board and the ethics committee of Basel, Switzerland.

Laboratory methods

Blood slides of all samples were examined for *P. falciparum* parasites using light microscopy and molecular methods as described previously (23,24). For molecular detection DNA was extracted from all blood samples using the QIAamp 96 DNA Blood kit (Qiagen; Mugil cohort) or the FavorPrep™ 96-well genomic DNA extraction kit (Favorgen; Maprik cohort). All samples were screened for the presence of *P. falciparum* using either PCR-ligase detection reaction fluorescent microsphere assay (PCR-LDR-FMA (26); Mugil cohort) or quantitative PCR (qPCR (27,28); Maprik cohort). In all samples positive for *P. falciparum* by any method, individual parasite clones were distinguished by amplifying the length-polymorphic *merozoite surface protein 2* (*msp2*) marker in nested PCR followed by high-resolution capillary electrophoresis (29,30). Electropherograms were analyzed using GeneMapper® software v3.7 (Applied Biosystems).

Estimating individual exposure

Individual exposure was calculated for each child from the number of new *P. falciparum* clones detected during follow-up divided by the individual time at risk (25) (chapter 3). A new infection was defined as an *msp2* allele not present in the two preceding genotyping-positive samples collected during active or passive surveillance without further allowing for imperfect detectability or clearance of clones (chapter 3).

Estimating clone detectability. infection duration and FOI

In order to estimate clonal detectability and compute detectability-adjusted FOI and clearance rates, the "triplet model" approach was used (4) (details presented in Supplement S1 Text). Briefly, presence and absence of each allele per child was recorded as a string of "1" (genotype present), "0" (genotype not present) or "?" (sample missing). To preserve the time intervals of sampling, data from unscheduled samples collected during passive case detection were merged to the closest active follow-up visit. A sliding window of three consecutive samples was passed along the string of 1's , 0's and ?'s, so that each sample could appear at any position in a triplet. Triplet patterns starting with a positive detection were counted. Estimates of detectability "S" and detectability-adjusted clearance rate per timestep "M" were computed using a Bayesian Markov chain Monte Carlo (MCMC) algorithm in WinBugs/JAGS software, assuming a Markov process for clearance and assuming the probability of re-infection with the same genotype within the same interval to be close to zero. Raw allele-specific incidence rates were calculated by dividing the number of gains (pattern "01", i.e. negative at t_1 and positive at t_2) by the number at risk for infection at time t_0 (patterns "00" and "01"), adjusted for M and S, and summed over alleles to obtain an detectability-adjusted incidence per time step "Λ". Lastly, M and Λ were transformed from transition probabilities to a continuous-time clearance rate "μ" and continuous-time aquisition rate "λ", i.e. FOI, with respect to the sampling interval used in the specific studies. In the Maprik cohort, sampling intervals differed between the first three months (2-weekly) and later five months (4-weekly) of the study, and thus an averaged interval timestep of 19.4 days was used.

To ensure that parameters reflected natural infections and were not biased by anti-malarial treatment, we excluded triplets that comprised an anti-malarial treatment. In a pilot sensitivity analysis, this strategy was compared to the full dataset and a restricted dataset excluding all triplets from children that received anti-malarial treatment. Estimates of detectability and infection duration were comparable using both exclusion strategies (Supplement S2 Table). Estimates of infection duration using the restricted datasets were longer than estimates obtained using the full dataset (Supplement S2 Table). Since the first strategy (excluding only those triplets that comprised treatment) allowed retaining more datapoints, this strategy was used for all further analyses.

Results

Comparing *P. falciparum* transmission between cohorts

P. falciparum transmission was higher in the Mugil cohort, performed 2004, than in the Maprik cohort, performed five years later. *P. falciparum* PCR prevalence at baseline, i.e. before the initial treatment, was 67% in Mugil and 24% in Maprik (Table 1). *P. falciparum* was the most common infection in Mugil but not in Maprik, where *P. vivax* was twice as prevalent (48%, Table 1). After the

respective initial anti-malarial treatment regimen (23,24), re-infection with *P. falciparum* occurred faster in the Mugil cohort (median time-to-reinfection: 55 days (23)) than in the Maprik cohort (median time to re-infection: >225 days (24)). In Mugil all but 31 (15%) of 206 children were re-infected during follow-up, as opposed to Maprik where more than two thirds of children (346/504) were not re-infected until the end of the study.

P. falciparum density by LM was 100-fold lower in Mugil versus Maprik (Table 1). In contrast, multiplicity of infection (MOI), i.e. the numbers of concurrent clones per sample, as well as the incidence of new clones in the blood were higher in Mugil than in Maprik (Table 1).

Table 1. Comparison of *Plasmodium* parasitological parameters between cohorts.

	Mugil cohort n=206 (CI_{95})	**Maprik cohort** n=504 (CI_{95})	***p*-value[1]**
Enrolment parameters			
P. falciparum PCR prevalence (%)	67 (61-74)	24 (20-28)	< 0.001
P. vivax PCR prevalence (%)	34 (28-41)	48 (43-52)	0.001
Parameters assessed during followup			
P. falciparum GMPD[2] by LM	12 (10-14)	1418 (1043-1928)	<0.001
Mean *P. falciparum* MOI[3] (clones/sample)	1.6 (1.6-1.7)	1.3 (1.3-1.4)	<0.001
Proportion of single-clone infections (%)	57 (54-60)	76 (72-80)	<0.001
Mean exposure (number of new clones/year[4])	6.4 (5.8-7.0)	4.1 (3.6-4.6)	<0.001

[1] *p*-values were calculated using Chi^2 test for differences in prevalence or proportions, and using Mann-Whitney -U test for differences in means.

[2] GMPD: geometric mean parasite density

[3] MOI, multiplicity of infection. Only *P. falciparum*-positive samples were included in calculation of the mean.

[4] Individual exposure calculated as the number of new clones/year observed in the blood stream (see methods). Mean was calculated including only children that were re-infected.

Triplet counts per study

Due to the higher number of *P. falciparum* infections in Mugil compared to Maprik, three times more triplets starting with a positive detection were counted in Mugil (Table 2). In addition, intermittent missed follow-visits were much more frequent in Maprik and 34% of triplets in the Maprik cohort included a missing sample (Table 2), leading to higher uncertainty of estimates obtained for this cohort.

Table 2. Triplet counts[1] by study.

	Mugil cohort		Maprik cohort	
Triplet	**Counts**	**% of triplets**	**Counts**	**% of triplets**
+ + X[2]	525	44.7	91	31.9
+ – +	61	5.2	22	7.7
+ – –	462	39.4	75	26.3
+ – ?	78	6.6	28	9.8
+ ? +	14	1.2	20	7.0
+ ? –	34	2.9	49	17.2
Total	**1174**	**100**	**285**	**100**

[1] Only triplets starting with a positive detection were counted.
[2] X: any of +, –, or ?

Detectability of *P. falciparum* clones

Overall, clone detectability was 78% (CI_{95}: 72-82) in Mugil and only 57% (CI_{95}: 43-70) in Maprik (Table 3). The effects of age and exposure, as surrogate markers of immunity, on clone detectability were investigated by forming age groups and groups of low and high exposure to *P. falciparum* within the two studies (Table 4). Age and exposure were not correlated in either study (Supplement S3 Figure, Gaussian generalized linear regression $p > 0.05$).

Table 3. Triplet model estimates by study.

Parameter	**Unit**	**Mugil cohort** Median (CI_{95})	**Maprik cohort** Median (CI_{95})
Detectability S	%	78 (72-82)	57 (43-70)
Adjusted acquisition rate λ	infections/year	14.4 (13.2-15.9)	3.3 (2.7-4.2)
Infection duration μ^{-1}	Days	38 (34-42)	62 (42-127)

In Maprik, where transmission was intermediate, clone detectability was lower in high-exposed children (50%, CI_{95}: 37-66) compared to low-exposed children (73%, CI_{95}: 49-90, Figure 1A). Clone detectability was slightly higher in older than in younger children in Maprik (49%, CI_{95}: 32-71; versus 61%, CI_{95}: 44-76, Figure 1A). Detectability declined with decreasing proportion of single-clone infections and with increasing MOI (Figure 1B&C). Clone detectability in Maprik was therefore lower the more concurrent clones were present.

In contrast, in the high-transmission setting of Mugil, detectability was equal in all subgroups despite variations in MOI and the proportion of single-clone infections (Figure 2A-C). In both studies, differences in parasite density between age and exposure groups were not pronounced, although in some cases significant (Figures 1D, 2D).

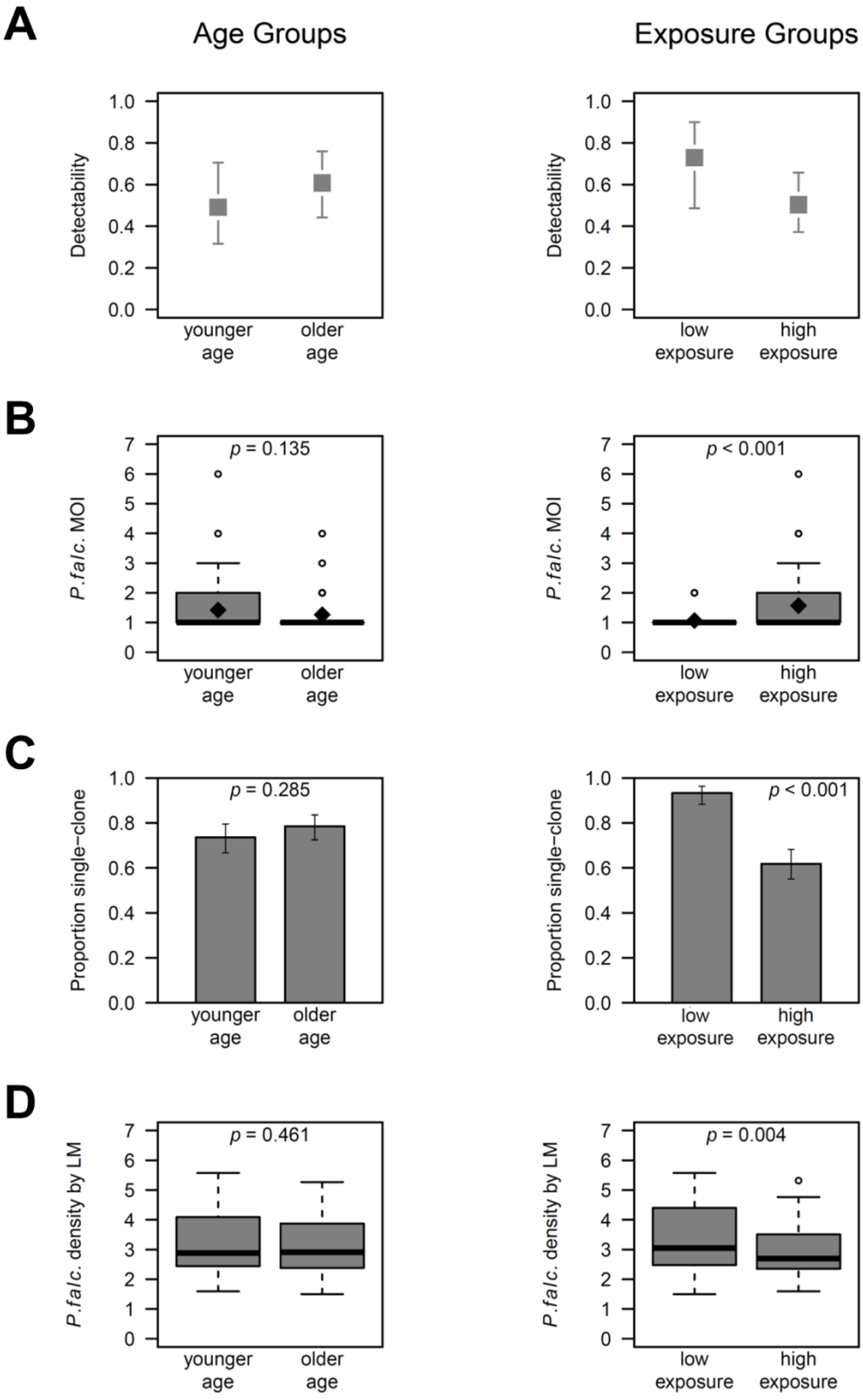

Figure 1. *P. falciparum* clone detectability and molecular parameters of infection by age or exposure group in the Maprik cohort. (A) Median detectability with 95% CI. (B) MOI by *msp2* genotyping; mean MOI is shown as black diamond. (C) Proportion of single clone infections among *P. falciparum*-positive samples by *msp2* genotyping. (D) Parasite density by LM. *p*-values were calculated using Mann-Whitney-U test (B, D) and Chi^2 test (C).

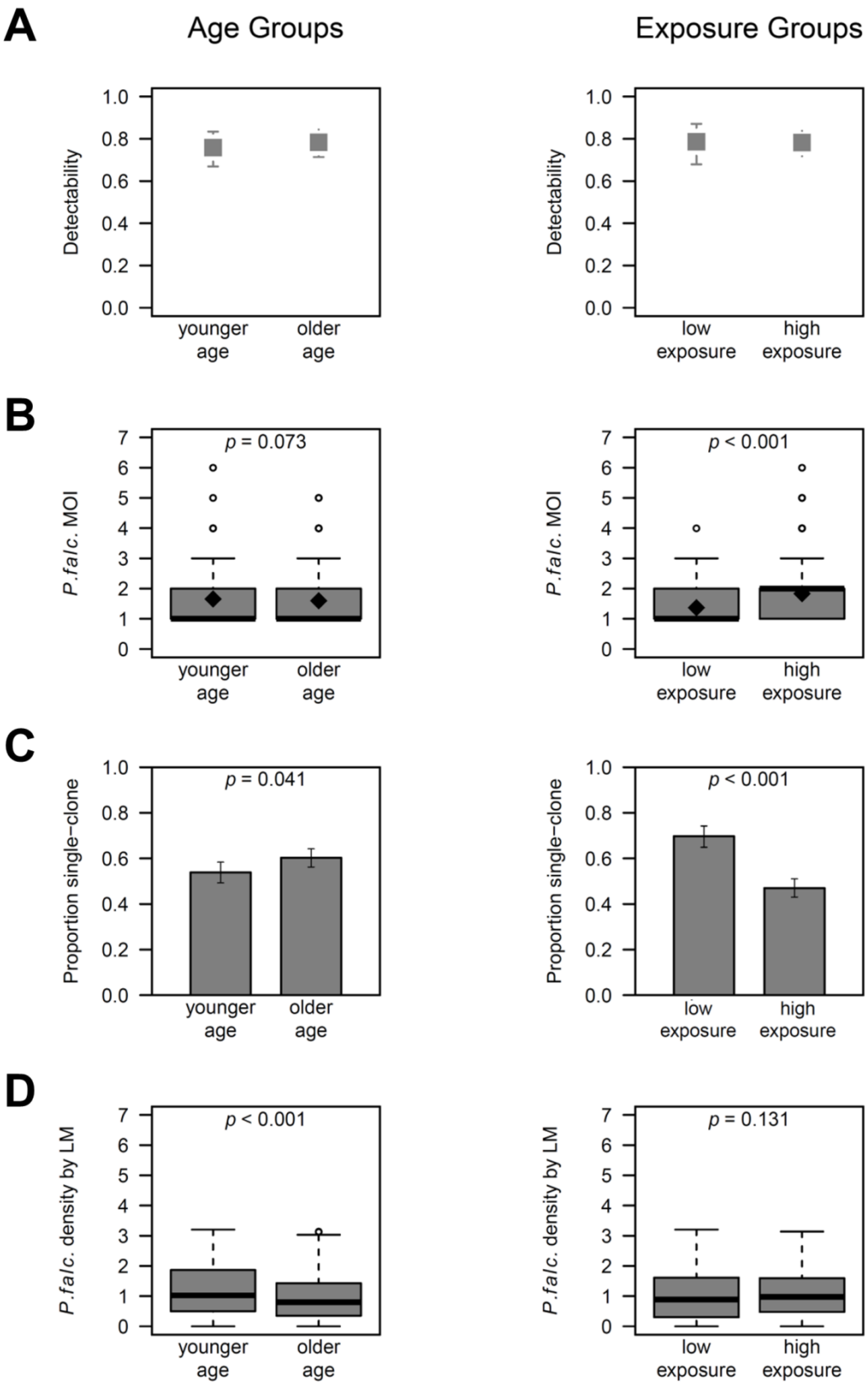

Figure2. *P. falciparum* clone detectability and molecular parameters of infection by age or exposure group in the Mugil cohort. (A) Median detectability with 95% CI. (B) MOI by *msp2* genotyping; mean MOI is shown as black diamond. (C) Proportion of single clone infections among *P. falciparum*-positive samples by *msp2* genotyping. (D) Parasite density by LM. *p*-values were calculated using Mann-Whitney-U test (B, D) and Chi^2 test (C).

Table 4. Characteristics of age groups and groups of low and high exposure by study.

	Mean (±StDev)	Number of children	Number of triplets[1]
Mugil cohort[2]			
Young	7.9 (±0.9) years	91	479
Old	10.5 (±1.2) years	115	695
Low-exposed	3.4 (±1.1) new clones/year	90[3]	363
High-exposed	9.6 (±3.2) new clones/year	85[3]	749
Maprik cohort[2]			
Young	6.2 (±0.7) years	245	127
Old	8.9 (±0.9) years	259	158
Low-exposed	2.2 (±0.7) new clones/year	101[3]	88
High-exposed	7.6 (±3.0) new clones/year	57[3]	190

[1] Only triplets starting with a positive detection were counted.
[2] Median age and median exposure per study were used as cutoff value to form groups of equal size.
[3] Children remaining uninfected throughout follow-up were not included in the exposure categories.

Duration of infection

In Mugil (high transmission), infections persisted on average only 38 days (CI_{95}: 34-42, Table 3). In Maprik (intermediate transmission), infections were slightly longer and lasted on average 62 days (CI_{95}: 42-127). The difference between studies was mainly due to long infection durations in highly exposed children in Maprik (98 days, CI_{95}: 51-713; Figure 3A), while infections in the low-exposed children in Maprik were as short as in children from Mugil (37 days, CI_{95}: 25-68). A longer duration of infection in higher exposed children was also observed in Mugil, but the difference was less pronounced compared to Maprik (low-exposed, 32 days, CI_{95}: 27-39; high-exposed, 42 days, CI_{95}: 37-49, Figure 3B). No pronounced trend in the duration of infection was observed between age groups (Figure 3).

Estimating FOI

Modeling the FOI, i.e. the acquisition rate of new infections, by adjusting for clone detectability and infection duration revealed a much larger difference in transmission between the two cohorts than anticipated based on means of individual exposure (Table 2, Table 3). Median modeled FOI was 14.4 infections/year (CI_{95}: 13.2-15.9) in Mugil compared to 3.3 infections/year in Maprik (CI_{95}: 2.7-4.2).

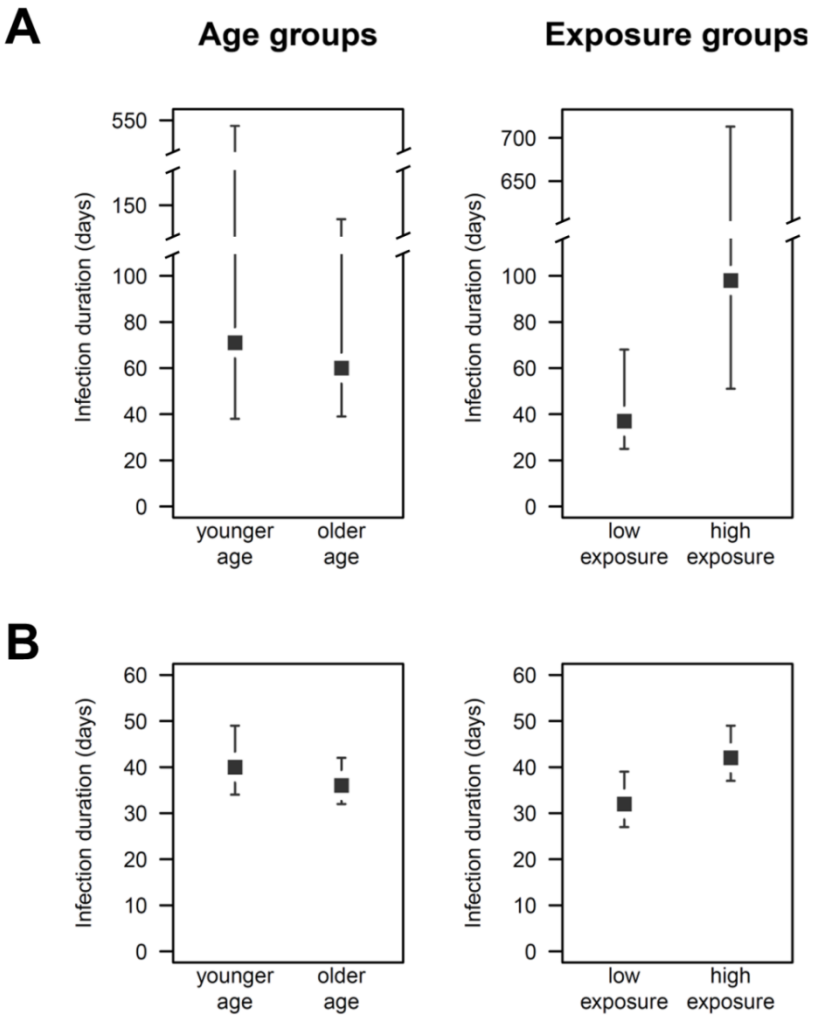

Figure 3. *P. falciparum* infection duration in the Maprik (A) and Mugil (B) cohorts. Median infection duration in days is shown by age group (left panel) and in low- versus high-exposed children (right panel). Error bars represent 95% CIs.

Discussion

This study contributed novel insights into the dynamic of non-African natural *P. falciparum* infections. In two cohort studies performed in PNG in settings of different transmission intensity, *P. falciparum* infection durations were significantly shorter than durations estimated using the same methodology in a previous cohort study in Ghana (31). Averaged over all ages, infections in the Ghanaian cohort lasted 168 days and even 216 days in 5-9 year-old children. In that cohort, sampling was performed in two-monthly intervals and clone detectability was much lower (40% in 5-9 year old children). Such large differences in the infection duration may reflect biological differences between African and PNG parasite populations, which face different environmental challenges. In Ghana, the

6-months long dry season is characterized by a virtually complete absence of rain (31), whereas seasonality is less pronounced in PNG and intermittent rainfall is frequent even in the dry season (32). While persistent chronic infections are thus required to re-initiate transmission after the dry season in many African settings (33), malaria transmission is perennial in PNG with seasonal fluctuations (32) and hence the selective pressure on the parasite population to maintain long-lasting chronic infections may be lower in PNG.

In addition, co-infection with other *Plasmodium* species, mainly with *P. vivax,* occurs frequently in PNG but less so in Africa and may have an impact on the duration of *P. falciparum* infections. While parasite density and the risk for malaria episodes are altered in mixed *P. falciparum*/*P. vivax* infections (chapter 3), we found no clear indication that differences in *P. vivax* carriage influenced *P. falciparum* infection durations (Supplement S4 Text). In Mugil, none of the assessed parameters of *P. falciparum* infection dynamics varied with *P. vivax* carriage (Supplement S4 Text). In Maprik, *P. vivax* carriage and exposure to *P. falciparum* were correlated, which prohibited investigating the effect of *P. vivax* carriage alone on infection duration (Supplement S4 Text).

Lastly, differences in parasite diversity between *P. falciparum* populations from Africa and PNG may contribute to the observed differences in infection duration. Recent analyses found a lower *P. falciparum var*-gene diversity in PNG compared to African populations (34,35), contrary to an earlier study describing equal *var*-gene repertoires (36). The ability of the parasite to switch to a *var*-gene variant previously not encountered by the host's immune system may hence be reduced in PNG compared to Africa, leading to earlier clearance.

The effect of acquired immunity on the duration of malaria infections is poorly understood, partly because a direct measure of protection is lacking. Age is widely used as surrogate marker (12) although a composite measure of age and exposure would more accurately reflect cumulative past exposure of an individual and hence immunity. No pronounced difference in infection duration was observed between age groups in the two PNG cohorts. Although the age range covered in both cohorts may be too small to detect significant age patterns, previous analyses demonstrated lower risk of *P. falciparum* episodes and high-density infections (23) as well as higher prevalence and titers of protective antibodies in children older than 9 years in the Mugil cohort (37–39). These data indicate that significant differences in immunity exist between the two age groups in the Mugil cohort. However, these differences did not have an impact on the duration of *P. falciparum* infections.

Longer infection duration observed in high-exposed compared to low-exposed children within both studies is therefore probably attributable to the interaction of concurrent clones and frequent super-infection, rather than differences in anti-malarial immunity. In contrast, an overall shorter infection duration was observed in Mugil (high transmission) compared to Maprik (intermediate transmission). This suggests that differences in immunity may play a role when drawing comparisons between

studies, where differences in immunity are supposedly larger than within a cohort. It is conceivable that children from Mugil, probably characterized by more advanced immunity owing to higher transmission, are better equipped to control several concurrent clones than children from Maprik, leading to shorter infection durations despite higher transmission.

In the Ghanaian cohort, clone detectability as well as parasite densities decreased with age (31). In PNG, detectability was not strongly related to overall parasite density but rather inversely correlated to MOI. This effect was stronger in intermediate-transmission Maprik than in high-transmission Mugil. One possible explanation may be a more advanced anti-malarial immunity of children in the Mugil cohort, who were almost constantly infected. As result they might be better able to control the density of superinfecting clones and to prevent expansion of a dominant clone in an already populated host, which is supported by the overall lower parasite densities (Figure 2D). Anti-malarial immunity in children from Maprik, in contrast, may still be incomplete and permit high density clones that could suppress minority clones. Concurrent clones may thus be more in numerical equilibrium and therefore easier detectable in children of the Mugil cohort compared to children from Maprik, despite the overall lower densities and higher MOI in Mugil.

When FOI was modeled including adjustments for clone detectability and clearance rate, it became evident that accurately estimating FOI is complex when sampling intervals approach the duration of infection. Median infection duration in Mugil was only 38 days and sampling was performed in monthly intervals. This suggests that a large proportion of infections in Mugil were only present in the host at one sampling visit, where they may or may not be detected. In contrast, sampling in Maprik was performed in two-weekly to monthly intervals and infections lasted on average 62 days. Infections were thus present in the host at up to four sampling visits in Maprik and were more likely to be detected compared to the Mugil cohort. As result, modeled FOI differed more drastically between studies than was anticipated based on the mean individual exposure, which does not take into account infection duration.

Because the primary objective of this analysis was assessing the duration of natural infections, we excluded triplets that included anti-malarial treatment when a child was symptomatic. Since malaria episodes are often caused by a new infection (40), we thereby selectively removed samples with a recent new *P. falciparum* infection. As result, the true FOI is likely even higher than the modeled FOI in both studies. Because more malaria episodes occurred in the Mugil compared to the Maprik cohort (23,24), this bias is expected to be more pronounced in Mugil. The true differences in exposure to new infections between the two studies may thus have been even larger than described by the modeled FOI.

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SUPPLEMENT

Supplement S1 Text:

Estimation of force of infection and clearance rates allowing for imperfect detectability

Analysis of the genotypes of malaria parasites in sequential blood samples collected several weeks apart from the same infected host, can be used to estimate the force of infection (FOI) of the parasite. Such data can also be used to estimate how long parasites persist if they are not treated. A significant complication is that even PCR does not detect all infections (1). This means that, when serial samples from the same host are considered, disappearance of a specific genotype in the second sample of a pair (a "loss" corresponding to a (+ −) pattern) can be either the result of imperfect detection of a persisting clone, or of clearance. Similarly, a "gain" (− +) can be the result of acquisition of parasites of the specific genotype, or of imperfect detection in the first sample of the pair.

The mathematics is best illustrated by the case of exchangeable genotypes, i.e. the rates of clearance of each genotype are the same and each genotype is equally detectible. In applications where this assumption does not hold, analyses can be carried out stratified by genotype or host characteristics. To estimate the rates of clearance allowing for the proportion of clones that are detected, sequences of at least three samples need to be considered. The following text is an expanded version of the original exposition of this method (2).

Clearance and detectability

To estimate the clearance rates and the detectability, a simple analytical approach is to group the data into "triplets" comprising sequences of three samples starting with each one that typed positive for each specific genotype, so that the total number of triplets is equal to the sum over all genotypes of the positive typings. These are then categorized according to the typing result and the presence of data for the second and third samples into categories (Figure 1 and Table 1).

The analysis estimates both the detectability and the probability that an infection existing at the start is cleared during the interval between the first sample (t=1) and the second sample (t=2) assuming a Markov process for clearance (2), and assuming the probability of reinfection within the same interval to be close to zero. The typing results at t=2 depend on probability, (M) , of clearance of an infection during any single interval and on the proportion of true infections detected in any given sample (S), as indicated in Table 1. In the case that the sample at t=2 is negative, the samples at t=3 are informative about whether a negative sample at t=2 was a true, or false negative. This method classifies pairs or triplets commencing with a positive sample into four categories (numbered 1-4 in Table 1 and Figure

1A), depending on the existence of data from samples at time t=2 and t=3. The likelihood of each of these 4 patterns, equivalent to the probability that it will be observed, conditional on the availability of the required typing data, is given in Table 1.

The original method (2) (Figure 1) analyses only the minimal dataset required to make the problem identifiable, counting only the pairs or triplets where samples are available at t=2, and distinguishing between cases where only pairs of samples (t=1 and t=2) are available from those where full triplets are available (t=1, t=2 and t=3). When the second sample is positive, the status at t=3 is not considered. The approach can be extended to incorporate information from all triplets with data from t=3, or from more complicated sample sets, including sequences of 4 or more samples and sequences with missing data. One such example is the case when the data at t=2 is missing (Figure S1B), but a sample at t=3 has been typed. Positivity at t=3 is then also indicative of persistence, and thus the t=3 provides some additional information about M and S. In the case that complete data are available from longer sample sets, the method is equivalent to that of Sama (3). The challenge of extending the methodology to analysis of longer series with complex patterns of missing data remains to be addressed.

Table 1. Patterns of infection detected by genotyping in sequences starting with positive samples, and their likelihoods

K	First sample	Second sample	Third sample	Likelihood
1	+	+	-, +, or missing	$L_1 = (1-M)S$
2	+	-	+	$L_2 = (1-M)^2(1-S)S$
3	+	-	-	$L_3 = 1 - L_2 - L_1$
4	+	-	Missing	$L_4 = 1 - L_1$
5	+	Missing	+	$L_5 = (1-M)^2 S$
6	+	Missing	-	$L_6 = 1 - L_5$

The values of *M* and *S* can be estimated by maximum likelihood from the overall likelihood constructed by multiplying the likelihoods for all the observed triplets, or equivalently from the log likelihood:

$$lnL = \sum_{k=1}^{4} n_k lnL_k$$

where n_k is the frequency of triplet pattern *k* (Table 1). Alternatively, a Bayesian Markov chain Monte Carlo (MCMC) algorithm can be employed using WinBugs or JAGS software assigning uniform priors to both *M* and *S* (see Appendix below). The decision points in Figure 1 then correspond to stochastic nodes with the probabilities for each branch proportional to the likelihoods from Table 1. The MCMC approach has the advantage of providing interval estimates for the parameters, and also allows the uncertainty in the estimation of M and S to be propagated in further calculations (e.g. bias-adjustments in estimates of FOI, see below).

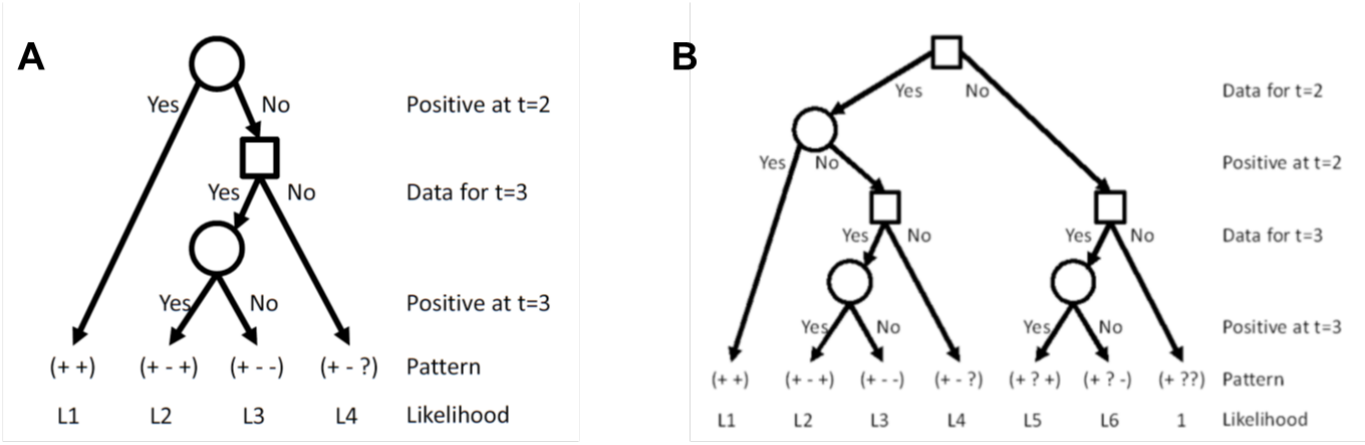

Figure 1. Classification of triplets, illustrated as branching process. The circles represent stochastic notes, the squares represent branching where the frequencies depend on whether samples are available, and thus do not provide information about infection dynamics. (A) Analysis including only pairs and triplets with data at t=2. (B) Analysis including triplets with missing data at t=2.

Infection/Acquisition probability

The acquisition probability for each genotype is estimated from the data of sequential pairs of samples, where the sequence starts with a sample that typed negative. The naïve estimate of the average number of infections acquired in each interval for genotype, *i*, is then:

$$\Lambda'_i = \frac{n_{-+}}{n_{--} + n_{-+}}$$

where n_{-+} is the total number of "gains" (− +) divided by the total intervals at risk, which is computed from sum of the number of "gains", n_{-+} , and n_{--} , the number of (− −) pairs for the genotype. This is a biased estimate of the true probability that there is a new infection during the interval, Λ_i, because of imperfect detection, which might affect either the first or second sample of the pair. The bias depends on *M*, *S*, and also on p_i, the true point prevalence of the genotype (i.e. the proportion of hosts infected by it on average at a single time point) (Table 2).

Table 2. Patterns of infections in sequences starting with samples testing negative for a specific genotype (*i*), and their probabilities of occurrence.

K	True pattern (T)	Probability that this pattern occurs	Probability that the first sample tests negative	Probability that this pattern occurs and the first sample tests negative (Pr(T))	Probability (− −) is observed $Pr(--\,|\mathrm{T}=k)$ if the first sample tests negative	Probability (− +) is observed $Pr(-+\,|\mathrm{T}=k)$ if the first sample tests negative
1	-	$(1-p_i)(1-\Lambda_i)$	1	$(1-p_i)(1-\Lambda_i)$	1	0
2	+	$(1-p_i)\Lambda_i$	1	$(1-p_i)\Lambda_i$	$1-S$	S
3	+ -	p_iM	$1-S$	$p_iM(1-S)$	1	0
4	+ +	$p_i(1-M)$	$1-S$	$p_i(1-M)(1-S)$	$1-S$	S

The overall probability that the observed pattern for genotype i in any sequential pair of samples is $(-\,-)$ is obtained as:

$$Pr(-\ -) = \sum Pr(\mathrm{T})Pr(-\ -\ \vert \mathrm{T} = \mathrm{k})$$

i.e.

$$Pr(-\ -) = (1-p_i)(1-\Lambda_i) + (1-S)(1-p_i)\Lambda_i + (1-S)p_iM + p_i(1-M)(1-S)^2,$$

and the overall probability that the observed pattern is (- +) is:

$$Pr(-\ +) = \sum Pr(\mathrm{T})Pr(-\ +\ \vert \mathrm{T} = \mathrm{k})$$

i.e.

$$Pr(-\ +) = S(1-p_i)\Lambda_i + p_i(1-M)(1-S)S.$$

In the large sample case, the observed proportion of intervals during which an infection appears to be acquired is then the ratio:

$$\Lambda'_i = \frac{Pr(-\ +)}{Pr(-\ -) + Pr(-\ +)}$$

which is therefore equivalent to:

$$\Lambda'_i = \frac{S\Lambda_i - Sp_i\big(\Lambda_i - (1-S)(1-M)\big)}{1 - Sp_i}$$

and,

$$\Lambda'_i = \frac{S\Lambda_i - p'_i\big(\Lambda_i - (1-S)(1-M)\big)}{1 - p'_i}$$

where: $p'_i = Sp_i$, is the point prediction of the observed prevalence which is estimated by:

$$\hat{p}'_i = \frac{n_+}{n_-\ +\ n_+}$$

n_- is the total number of samples testing negative for the genotype, and n_+ is the total testing positive. Solving for Λ_i and substituting the field-based estimates, provides a formula for the bias adjusted estimate of Λ_i as a function of $\hat{p}'_i$ and of the estimated detectability, $\hat{S}$, and clearance probability, $\widehat{M}$.

$$\hat{\Lambda}_i = \frac{\Lambda'_i - \hat{p}'_i\left(\Lambda'_i + \big(1-\hat{S}\big)\big(1-\widehat{M}\big)\right)}{\hat{S} - \hat{p}'_i}$$

This is a correction to the formula originally published in 1999 (2).

The ratio, $\hat{\Lambda}_i/\Lambda'_i$ which measures the bias in the crude estimate of the number of new infections, is highly sensitive to $\hat{S}$. If detection is perfect, it has a value of 1.0, corresponding to no bias. At low values of $\hat{S}$, $\hat{\Lambda}_i$ can be substantially higher than Λ'_i especially if $\widehat{M}$ is high, corresponding to long survey intervals or rapid turnover of infections (Figure 2). Within the ranges observed in field studies in Tanzania and Ghana (2,3) the ratio of $\hat{\Lambda}_i/\Lambda'_i$ is a non-monotonic function of $\hat{S}$ but remains fairly close to unity (implying only modest levels of bias). The bias is very insensitive to $\hat{p}'_i$ over a wide range of prevalence values. In general, since the analysis is applied to genotype specific data, $\hat{p}'_i$ is expected to be low.

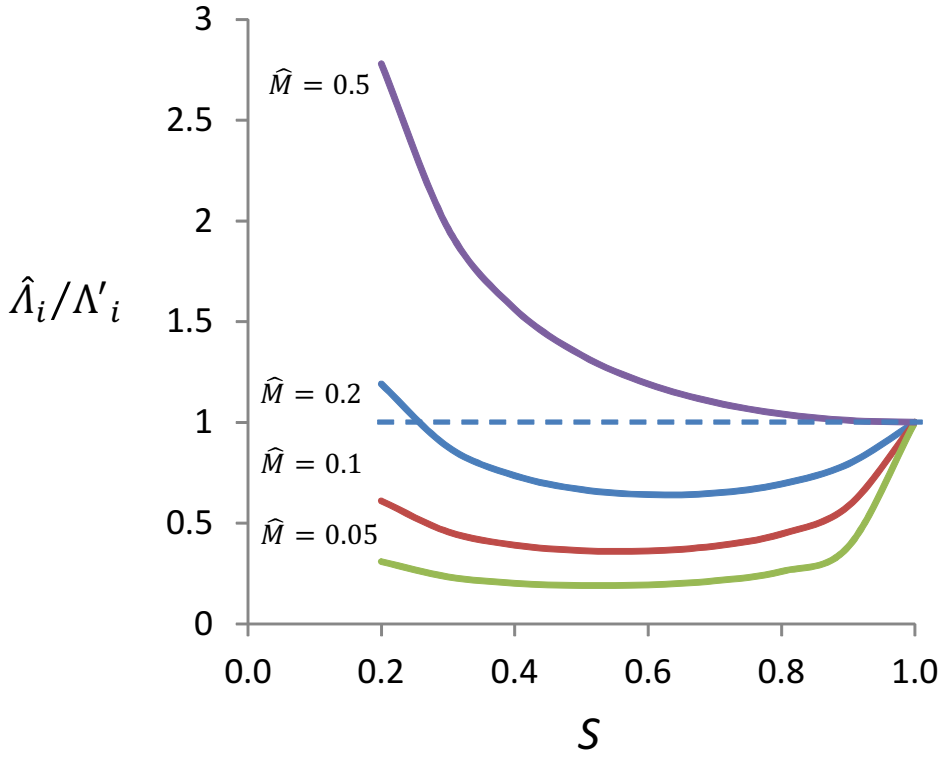

Figure 2. Bias in estimated FOI. The quantity plotted is the ratio of the true value to the unadjusted estimate in each case for $\hat{p}'_i = 0.002$. The horizontal dashed line corresponds to the case of no bias.

Conversion to rates

$\widehat{M}$ and $\hat{\Lambda}_i$ are estimates of transition probabilities, and are therefore specific for the interval length used in any specific study. These must be converted to rates in continuous time if they are to be compared across studies with different interval durations. The continuous time analogues correspond to the reversible catalytic model (4,5). This provides expressions for transition probabilities as functions of the rates:

$$\hat{\Lambda}_i = \frac{\hat{\lambda}_i}{\hat{\lambda}_i + \hat{\mu}_i}\left[1 - exp\left(-\left(\hat{\lambda}_i + \hat{\mu}_i\right)t\right)\right]$$

and:

$$\widehat{M} = \frac{\widehat{\mu_\iota}}{\hat{\lambda}_i + \widehat{\mu_\iota}}\left[1 - exp\left(-\left(\hat{\lambda}_i + \widehat{\mu_\iota}\right)t\right)\right]$$

where t is the duration of the inter-survey interval, $\hat{\mu}$ is the estimate of the clearance rate, and $\hat{\lambda}_i$ is the estimate of the genotype specific FOI. Rearrangement of these formulae and solving for $\widehat{\mu_\iota}$ and $\hat{\lambda}_i$ gives:

$$\widehat{\mu_\iota} = \frac{-\widehat{M} ln\left(1 - \left(\hat{\Lambda}_i + \widehat{M}\right)\right)}{\left(\hat{\Lambda}_i + \widehat{M}\right)t}$$

and:

$$\hat{\lambda}_i = \frac{\hat{\Lambda}_i}{\widehat{M}}\widehat{\mu_\iota}$$

The overall FOI is estimated as the sum of the individual $\hat{\lambda}_i$ values. Although we use a common value of $\widehat{M}$ across all genotypes, this analysis leads to slightly different values of $\widehat{\mu_\iota}$. The mean of these calculated allele-specific values is reported as the clearance rate, $\bar{\mu}$. The reported average duration of infection in days is then proportional to the reciprocal of this value, $1/\bar{\mu}$.

Interval estimation

Estimation of FOI, clearance rates, and detectability can be carried out by Maximum Likelihood, or alternatively by Bayesian MCMC. The latter has the advantage that it provides sampling-based interval estimates for all the parameters, potentially allowing for the uncertainties at each stage of the algorithm. Code for MCMC estimation is appended.

WinBugs/JAGS code for estimating $\widehat{M}$, S, $\hat{\lambda}_i$, $\hat{\mu}_i$.

 # IMPLEMENTATION OF THE MODEL OF FIGURE S1A WITH DETERMINISTIC ESTIMATION OF THE FORCE OF INFECTION

```
model {
#ESTIMATION OF M AND S
  L1<- (1-M)*S
  L2<- (1-M)*(1-M)*(1-S)*S
  L3<- 1 - L1- L2
  L4<- 1 - L1

#
  pr2<- L2/(L2+L3)
  s1total <- n11+n101+n100+n10
  s2total <- n101+n100

# binomial sampling of stochastic nodes 1, 2
  n11  ~ dbin(L1,s1total)
  n101 ~ dbin(pr2,s2total)

# uniform prior for M and S
  M ~ dunif(0,1)
  S ~ dunif(0,1)

#ESTIMATION OF FORCE OF INFECTION AND BIAS ADJUSTMENT
 for (i in 1:NumberOfGenotypes)
   {
   L[i] <- (lambdaPrime[i] - p[i]*(lambdaPrime[i] + (1-M)*(1-S)))/(S -
p[i])
   p[i] <- n1[i]/n[i]
   lambdaPrime[i] <- n01[i]/n0[i]

# transform to continuous time
   muDaily[i]<- -M*log(1-(L[i]+ M))/((L[i]+M)*t)
   lambdaDaily[i]<- -L[i]*log(1 - (L[i]+M))/((L[i]+M)*t)
 }

#summaries over all genotypes
ClearanceDaily<-mean(muDaily[])
FOIDaily<-sum(lambdaDaily[])
}


# TEST DATA

list(NumberOfGenotypes=7, n11=211, n101=61, n100=209,n10=71,t=14)

n0[]  n01[] n[]   n1[]
560   11    3000  53
560   18    3000  57
560   10    3000  43
560   9     3000  23
```

```
560   11    3000  56
560   15    3000  72
560   5     3000  15
END

# EXAMPLE INITIAL VALUES (1 CHAIN)
list(
M = 0.1440881879701678,
S = 0.4112187770372916,
logitlambdaPrime = c(
-4.207892579282936,-4.168605593430168,-3.965568672495186,-
4.388163834738868,-3.892549139358283,
-3.908037902822194,-5.066532048318275),
logitlambdaPrimebar = -3.823480945836294,
logitp = c(
-4.086307570550217,-4.004132629521775,-4.390522771059175,-
4.994397880509394,-3.750283683731104,
-3.892052972624451,-5.191441694450187),
logitpbar = -4.172449215059147,
tau = 2.818547930754359,
taup = 2.08985431148514)
```

 # IMPLEMENTATION OF THE MODEL OF FIGURE S1B WITH STOCHASTIC ESTIMATION OF THE FORCE OF INFECTION

```
model {
#ESTIMATION OF M AND S
  L1<- (1-M)*S
  L2<- (1-M)*(1-M)*(1-S)*S
  L3<- 1 - L1- L2
  L4<- 1 - L1
  L5<- (1-M)*(1-M)*S
  L6<- 1 - L5
#
  pr2<- L2/(L2+L3)
  pr3<- L5/(L5+L6)
  s1total <- n11+n101+n100+n10
  s2total <- n101+n100
  s3total <- n1_1+n1_0

# binomial sampling of stochastic nodes 1, 2 and 3
  n11  ~ dbin(L1,s1total)
  n101 ~ dbin(pr2,s2total)
  n1_1 ~ dbin(pr3,s3total)

# uniform prior for M and S
  M ~ dunif(0,1)
  S ~ dunif(0,1)

#ESTIMATION OF FORCE OF INFECTION AND BIAS ADJUSTMENT
 for (i in 1:NumberOfGenotypes)
   {
   L[i]<- (lambdaPrime[i] - p[i]*(lambdaPrime[i] + (1-M)*(1-S)))/(S - p[i])
   n1[i]~dbin(p[i],n[i])
   n01[i]~dbin(lambdaPrime[i],n0[i])
```

```
# random effects for genotype specific prevalence and incidence
   lambdaPrime[i]<- 1/(1+exp(-logitlambdaPrime[i]))
   logitlambdaPrime[i]~dnorm(logitlambdaPrimebar,tau)
   p[i]<- 1/(1+exp(-logitp[i]))
   logitp[i]~dnorm(logitpbar,taup)

# transform to continuous time
   muDaily[i]<- -M*log(1-(L[i]+ M))/((L[i]+M)*t)
   lambdaDaily[i]<- -L[i]*log(1 - (L[i]+M))/((L[i]+M)*t)
 }

#priors for metaparameters of random effects
logitlambdaPrimebar~dnorm(0,1.0E-2)
tau~dunif(0,10)
logitpbar~dnorm(0,1.0E-2)
taup~dunif(0,10)

#summaries over all genotypes
ClearanceDaily<-mean(muDaily[])
FOIDaily<-sum(lambdaDaily[])
}


# TEST DATA

list(NumberOfGenotypes=7, n11=211, n101=61,
n100=209,n10=71,n1_0=30,n1_1=10,t=14)

n0[]  n01[] n[]   n1[]
560   11    3000  53
560   18    3000  57
560   10    3000  43
560   9     3000  23
560   11    3000  56
560   15    3000  72
560   5     3000  15
END

# EXAMPLE INITIAL VALUES (1 CHAIN)

list(
M = 0.2938271930359447,
S = 0.5093814682038473,
logitlambdaPrime = c(
-3.562748062328747,-3.678312884623953,-3.841609547868719,-
4.561559522968858,-3.54204822963872,
-3.543603660614651,-4.123941440039693),
logitlambdaPrimebar = -3.637727825838637,
logitp = c(
-4.164460955662759,-4.017057261011612,-4.191668882209104,-
4.558031283327824,-3.778424732596439,
-3.785125363187561,-4.934854572547822),
logitpbar = -4.653282454126015,
```

```
tau = 2.097024953285597,
taup = 2.599193884837493)


# M,S, ClearanceDaily and FOIDaily are the main parameters of interest.
```

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Supplement S2 Table:

Table S2. Comparison of strategies to account for anti-malarial treatment in the Maprik cohort.

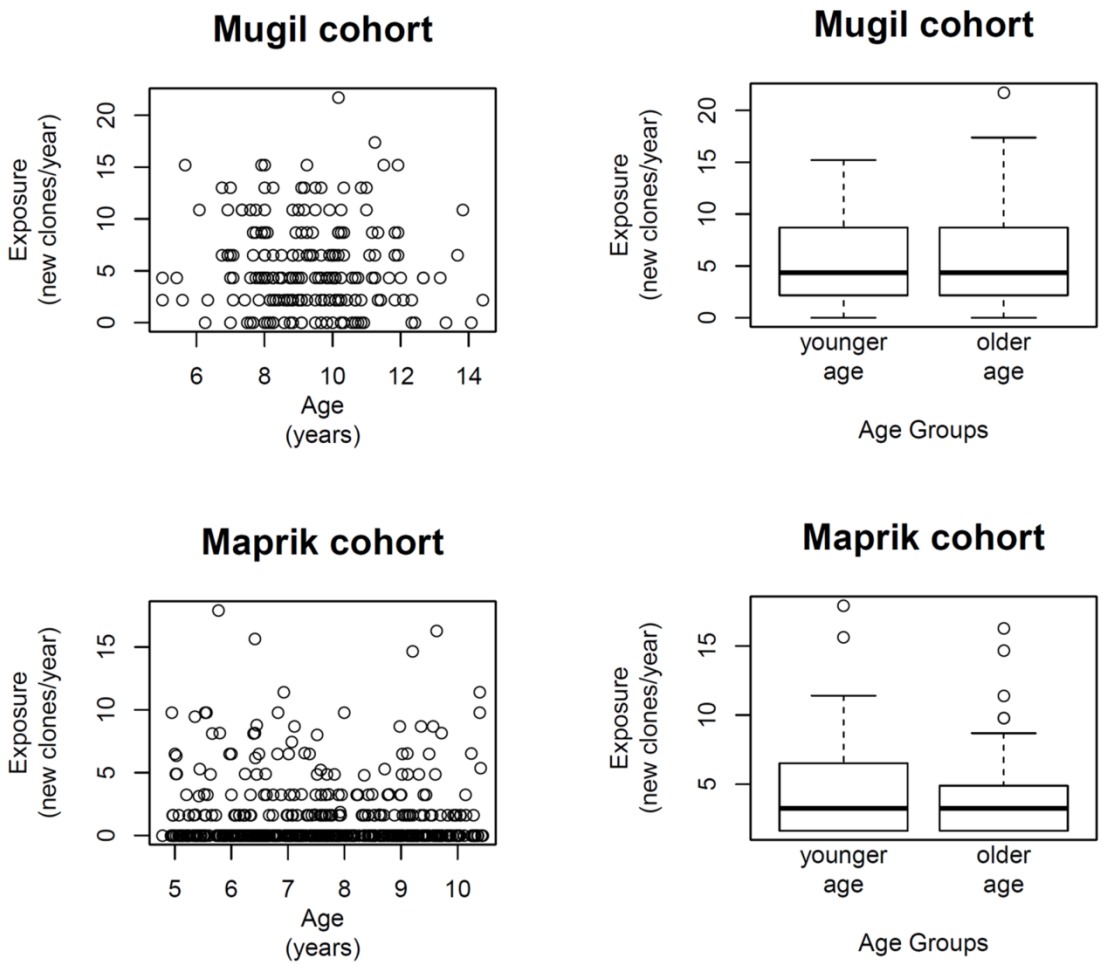
	Full dataset	**Strategy 1** Exclusion of children that received anti-malarial treatment	**Strategy 2** Exclusion of triplets that comprised anti-malarial treatment
Number of children	504	374	504
Number of triplets[1]			
Total	**351**	**230**	**285**
+ + X[2]	100	79	91
+ – +	23	18	22
+ – –	120	53	75
+ – ?	36	26	28
+ ? +	20	17	20
+ ? –	52	37	49
	Median (CI_{95})	Median (CI_{95})	Median (CI_{95})
Detectability S	55 (41-68)	58 (44-72)	57 (43-70)
Adjusted acquisition rate λ (infections/year)	3.8 (3.1-4.8)	2.8 (2.2-3.6)[3]	3.3 (2.7-4.2)[3]
Infection duration μ^{-1} (days)	47 (35-80)	69 (45-176)	62 (42-127)

[1] Only triplets starting with a positive detection are counted.

[2] X: any of +, –, or ?

[3] Exclusion of children that received anti-malarial treatment, or exclusion of triplets that comprised anti-malarial treatment introduces bias towards lower acquisition rates since highly exposed children, or time points of new infections, are selectively removed. Lower acquisition rates compared to the full dataset are therefore to be expected.

Supplement S3 Figure:



S3 Figure: Age and exposure are not correlated in the Mugil and Maprik cohorts. Gaussian generalized linear regression revealed no significant associations between age and exposure (Mugil p=0.376; Maprik p=0.452)

Supplement S4 Text:

Investigating the effect of *P. vivax* co-infection on *P. falciparum* infection duration

.

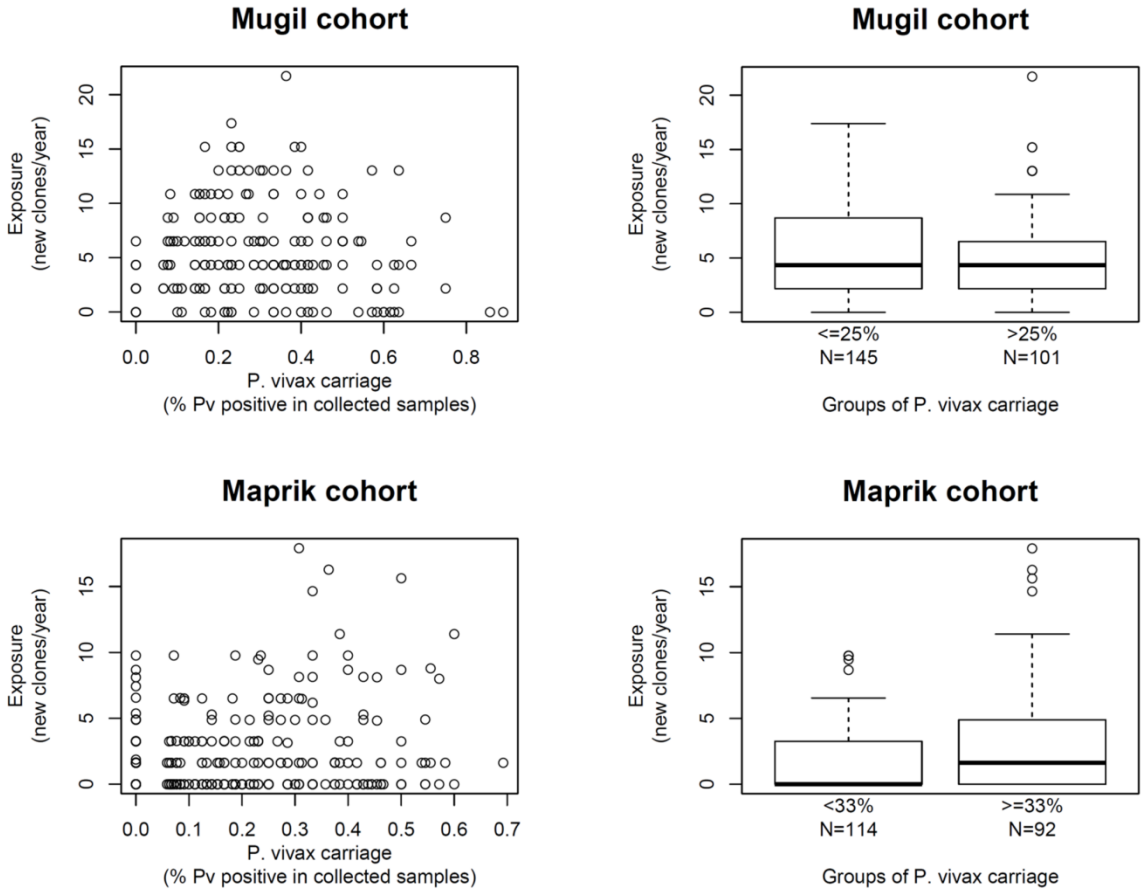


Figure 1. *P. vivax* co-infection and exposure to *P. falciparum* are highly correlated in the Maprik cohort but not the Mugil cohort. Exposure to *P. falciparum* is plotted against *P. vivax* carriage per child. Individual exposure to *P. falciparum* was calculated as described in the methods section (y-axis). *P. vivax* carriage was calculated as the proportion of *P. vivax* qPCR-positive samples in all samples collected per child (x-axis). The median *P. vivax* carriage per study was used to define the cutoff between groups of low and high *P. vivax* carriage. Association of *P. vivax* carriage and exposure to *P. falciparum* was assessed using Gaussian generalized linear models. A significant association was found for the Maprik cohort ($\beta = 5.95$ [CI_{95}: 4.59 - 7.31], $p<0.001$), but not the Mugil cohort ($\beta = -0.81$ [CI_{95}: -4.05 - 2.42], $p=0.622$).

Table 1. The apparent effect of *P. vivax* carriage on *P. falciparum* infection durations in the Maprik cohort is likely an artifact caused by the correlation of *P. vivax* carriage with exposure to *P. falciparum*. Differing *P. vivax* carriage has no effect on *P. falciparum* infection durations in the Mugil cohort. In the Maprik cohort, patterns in triplet model outcomes between groups of low and high *P. vivax* carriage match those between *P. falciparum* exposure categories. The median *P. vivax* carriage per study was used to define the cutoff between groups of low and high *P. vivax* carriage.

	Detectability S (%) Median (CI_{95})	**Infection duration μ^{-1} (days)** Median (CI_{95})	**Adjusted acquisition rate (λ)** Median (CI_{95})
Mugil cohort			
P. vivax carriage			
Low	79 (72-85)	41 (36-48)	14.7 (13.2-16.7)
High	76 (67-83)	34 (29-41)	14.2 (12.4-16,6)
P. falciparum exposure			
Low	79 (72-85)	32 (27-39)	9.6 (8.2-11.5)
High	76 (67-83)	42 (37-49)	22.5 (20.2-25.3)
Maprik cohort			
P. vivax carriage			
Low	63 (44-80)	**48 (32-103)**	1.8 (1.4-2.6)
High	51 (36-69)	**88 (46-660)**	10.6 (7.9-14.1)
P. falciparum exposure			
Low	73 (49-90)	**37 (25-68)**	4.1 (3.1-6.1)
High	50 (37-66)	**98 (51-713)**	28.9 (21.6-38.8)

Chapter 5:

P. vivax and *P. falciparum* gametocyte carriage after radical cure

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RESEARCH ARTICLE

Effects of liver-stage clearance by Primaquine on gametocyte carriage of *Plasmodium vivax* and *P. falciparum*

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Abstract

Background

Primaquine (PQ) is the only currently licensed antimalarial that prevents *Plasmodium vivax* (*Pv*) relapses. It also clears mature *P. falciparum* (*Pf*) gametocytes, thereby reducing post-treatment transmission. Randomized PQ treatment in a treatment-to-reinfection cohort in Papua New Guinean children permitted the study of *Pv* and *Pf* gametocyte carriage after radical cure and to investigate the contribution of *Pv* relapses.

Methods

Children received radical cure with Chloroquine, Artemether-Lumefantrine plus either PQ or placebo. Blood samples were subsequently collected in 2-to 4-weekly intervals over 8 months. Gametocytes were detected by quantitative reverse transcription-PCR targeting *pvs25* and *pfs25*.

Results

PQ treatment reduced the incidence of *Pv* gametocytes by 73%, which was comparable to the effect of PQ on incidence of blood-stage infections. 92% of *Pv* and 79% of *Pf* gametocyte-positive infections were asymptomatic. *Pv* and to a lesser extent *Pf* gametocyte positivity and density were associated with high blood-stage parasite densities. Multivariate analysis revealed that the odds of gametocytes were significantly reduced in mixed-species infections compared to single-species infections for both species (OR_{Pv} = 0.39 [95% CI 0.25–0.62], OR_{Pf} = 0.33 [95% CI 0.18–0.60], p<0.001). No difference between the PQ and placebo treatment arms was observed in density of *Pv* gametocytes or in the proportion of *Pv* infections that carried gametocytes. First infections after blood-stage and placebo treatment,

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Competing interests: The authors have declared that no competing interests exist.

likely caused by a relapsing hypnozoite, were equally likely to carry gametocytes than first infections after PQ treatment, likely caused by an infective mosquito bite.

Conclusion

Pv relapses and new infections are associated with similar levels of gametocytaemia. Relapses thus contribute considerably to the Pv reservoir highlighting the importance of effective anti-hypnozoite treatment for efficient control of Pv.

Trial registration

ClinicalTrials.gov NCT02143934

Author summary

Plasmodium vivax (*Pv*) mainly affects Asia, Central and South America as well as Ethiopia. In Papua New Guinea (PNG) *Pv* prevalence is among the highest worldwide. The biggest challenge for the control of *Pv* infections is the formation of dormant liver stages, which have the ability to relapse and cause disease even after successful clearance of asexual stages in the blood circulation. Primaquine is the only licensed drug that is able to prevent *Pv* relapses. A randomized treatment-to-reinfection cohort in Papua New Guinean children permitted permitted the study of *Pv* and *P. falciparum* gametocyte carriage after radical cure with Primaquine and to investigate the contribution of *Pv* relapses to transmission. We found that most gametocyte carriers in this study were detected in asymptomatic infections and that relapses and new infections are associated with similar *Pv* gametocyte production. These are strong arguments emphasizing the importance of sensitive detection and early treatment of asymptomatic and submicroscopic Plasmodium *spp.* infections and of anti-hypnozoite treatment for an effective control of *Pv*.

Introduction

Primaquine (PQ) is the only currently licensed drug for preventing *Plasmodium vivax* (*Pv*) relapses [1], and also the only effective drug against mature gametocytes of *P. falciparum* (*Pf*) [2,3]. Since 2012, the World Health Organization recommends a single dose of PQ for treatment of *Pf* infections with the aim to reduce post-treatment *Pf* gametocyte carriage and thus the potential for onward malaria transmission [4].

Gametocyte development as well as morphology differs considerably between *Pv* and *Pf* [5]. *Pv* gametocytes mature rapidly and are detectable in the peripheral blood as early as two or three days following detection of blood-stage parasites by qPCR or light microscopy (LM), respectively [6,7]. In contrast, *Pf* gametocytes sequester for 7–10 days in the bone marrow before being released into the blood circulation [8], where they are observed by LM 10–15 days after the first detection of asexual parasites [9]. Gametocytes were observed in symptomatic *Pv* episodes at higher frequency compared to *Pf* episodes, despite 10-fold lower *Pv* blood-stage densities compared to *Pf* [10]. After drug treatment, *Pv* gametocytes are cleared within days after clearance of blood-stage infections in contrast to *Pf* gametocytes, which circulate over 3 weeks following successful blood-stage clearance [6,9,11,12]. Altogether the published data suggests that *Pv* infections produce proportionally higher gametocyte densities than *Pf*

infections (at the same levels of asexual parasitaemia), and that *Pv* gametocytes mature more rapidly [9,12–14].

Not much is known about gametocyte production in primary *Pv* infections versus relapses from activated hypnozoites, mainly because in endemic settings it is impossible to distinguish both sources of infection. Our previous work in Papua New Guinea (PNG) showed that relapsing *Pv* infections contributed 73% of the gametocyte carriage [15]. A study in Thailand and Indonesia reported that densities by LM of *Pv* blood-stage parasites and gametocytes were similar in new infections and relapses [16]. Both studies indicated the need for efficient treatment of the hypnozoite reservoir for reducing *Pv* transmission [15,16].

A challenge in studying the investment of *Pv* infections in gametocytogenesis is the generally low and often submicroscopic density of asexual parasites and gametocytes. In addition, scarce *Pv* gametocytes can easily be misclassified by LM due to their resemblance to late trophozoites [17]. Investigating gametocyte production of *Pv* infections hence requires sensitive and specific molecular methods. For *Pf*, studying gametocytes by LM is more feasible because of the distinct crescent-shaped morphology of gametocytes and generally higher parasite densities; however also for *Pf*, molecular methods are crucial for studying gametocytes in low-density *Pf* infections. Molecular detection of gametocytes usually targets transcripts of the *Pf* or *Pv* 25 kDa ookinete surface antigen precursor (*pfs25* or *pvs25*, respectively) [18,19], which are highly expressed in mature gametocytes [20,21]. Expression of the *pfs25* transcripts is mainly female specific, hence male gametocytes are detected to a much lower extend by *pfs25*-based assays [22]. Female gametocytes are generally over-represented in peripheral blood samples with about 3.5 female per each male gametocyte [23,24]. It can therefore be estimated that *pfs25* RT-qPCR assays detect approx. 70% of the total number of gametocytes. Both *pfs25* and *pvs25* quantitative reverse transcription PCR (qRT-PCR) or nucleic acid sequence-based amplification (NASBA) can detect as few as 1 *Pf* gametocyte or 10 *Pv* gametocytes per 50 μl blood and are therefore up to 50x more sensitive than LM [18,19,25].

This study investigated gametocyte dynamics of *Pf* and *Pv* infections in school-aged PNG children after randomized treatment with blood-stage antimalarials plus PQ or placebo. This trial design permitted an evaluation of the contribution of hypnozoites to *Pv* infection parameters by comparison of two treatment arms. PQ treatment for clearance of hypnozoites reduced the risk of recurrent *Pv* blood-stage infection by 82% [95% CI 0.75–0.86], the risk of a *Pv* episode by 75% [95% CI 0.49–0.89], and the incidence of *Pv* gametocytes by 73% [95% CI 0.62, 0.81][15]. Another study conducted in Thailand and Indonesia revealed that density of gametocytes over time followed that of asexual parasites [16]. However, the Thai-Indonesian study depended on the presentation of patients at a health facility upon occurrence of a clinical episode and did not use molecular methods to detect submicroscopic asexual parasites or gametocytes [16]. Our initial report on the PNG cohort study is now extended to address the following questions: (i) what are risk factors for *Pf* and *Pv* gametocyte carriage? (ii) does gametocytaemia differ between *Pv* new infections and relapses? And (iii) does PQ treatment exert a long-term effect on *Pf* and *Pv* gametocytaemia?

Methods

Study design

The study was conducted in 2009 to 2010 in the Albinama area, East Sepik province, in PNG. A detailed study protocol has been published previously [15]. In brief, 504 children aged 5 to 10 years were randomized to two treatment arms and completed directly observed treatment (DOT) with a 3-day dose of Chloroquine (CQ), a 3-day dose of Artemether-Lumefantrine (AL) and either 20 doses of PQ (per day: 0.5 mg/kg) or placebo over four weeks. Children were

screened for G6PD deficiency by using a visual colorimetric method (G6PD Assay Kit WST-Dojindo Co., Japan). Venous blood samples were collected at enrolment (prior to treatment) and 3 days after the final dose of DOT. The latter date represented day 0 of follow-up. Finger-prick samples were taken every two weeks for the first 3 months and monthly for the remaining 5 months of follow-up. Symptomatic children detected during follow-up were treated with a 3-day course of AL after confirming *Plasmodium* infection by rapid diagnostic test (RDT, CareStart Malaria pLDH/HRP2 Combo, AccessBio, USA).

Ethics statement. The study received ethical clearance by the PNG Institute of Medical Research (IMR) Institutional Review Board (0908), the PNG Medical Advisory Committee (09.11), the Ethikkommission beider Basel (237/11) and was registered on ClinicalTrials.gov NCT02143934. A parent or guardian of every child participant provided written informed consent for their participation.

Detection of blood-stage parasites and gametocytes

All blood samples collected were examined by LM and quantitative PCR (qPCR). Blood slides were examined by at least two independent microscopists and declared parasite negative only after examination of 200 thick-film fields [15]. Parasite DNA was extracted from 100–150 μl blood cell pellet using the FavorPrep 96-well genomic DNA extraction kit (Favorgen, Taiwan) and analyzed for *Pf* and *Pv* positivity by *18S rRNA* qPCR [15,19]. All *Pv* and *Pf* qPCR positive samples were genotyped using markers *Pv-msp1*F3 and *Pf-msp2*, respectively, following previously published protocols [26,27].

RNA was extracted from all samples positive in *Pf* or *Pv* qPCR. RNA was extracted using the RNEasy 96 kit (Qiagen, Switzerland) as described previously [19] from 50μl whole blood spotted on filter papers that had been air-dried and stored in TRIzol reagent (Life Technologies, Switzerland). Gametocyte-specific transcripts were detected by *pfs25* or *pvs25* qRT-PCR [19] in all RNA samples for which the corresponding DNA sample had been positive by species-specific qPCR.

Statistical analysis

Children were censored on the last visit before two consecutively missed scheduled follow-up visits [15]. Comparison of LM-positive versus submicroscopic infections, and symptomatic versus asymptomatic infections was performed with 5019 samples from the follow-up period for which LM data was available. A clinical malaria episode was defined as fever (axillary temperature >37.5˚C and/or fever reported in previous 2 days) and the presence of *Plasmodium spp*. parasites by LM. Differences in proportions were tested for statistical significance using the McNemar X^2 test with continuity correction. To achieve normal distribution, qPCR densities were expressed as $\log_{10}$-transformed *18S rRNA* genomic copies/μl blood for asexual parasites, and $\log_{10}$-transformed *pfs25* or *pvs25* transcripts/μl blood for gametocytes. Correlation between microscopic parasite counts and molecular methods was tested by Kendall's rank sum test on $\log_{10}$ transformed data. Geometric means of densities were calculated. Differences in densities of asexual or sexual-stage parasites were tested for statistical significance using Welch's Two-sample t-test.

Negative binomial regression models were used to calculate the incidence rate of *Pv* and *Pf* gametocyte positivity as previously described [15]. Gametocyte positivity during follow-up was modeled using binomial generalized estimating equations (GEE) with logit link using an exchangeable correlation matrix to account for repeated measures by child. Log_{10}-transformed blood-stage parasite density and gametocyte density during follow-up were modeled using Gaussian GEEs with log link using an exchangeable correlation matrix. Linear fit for $\log_{10}$-

transformed blood-stage parasite density was previously analyzed and considered adequate for both species (S1 Text). All Models were back-selected. Statistical analyses were conducted using R version 3.1.1 [28] or STATA version 14.

Results

Gametocyte positivity and density in submicroscopic infections

Molecular methods were superior to LM especially for detection of gametocytes but also for blood-stage parasites (Fig 1). By LM *Pv* gametocytes were detected in only 44 out of 366 *Pv* positive samples (12%), whereas by molecular detection 265 out of 705 *Pv* samples (38%, p<0.001) were gametocyte-positive. *Pf* gametocyte rates by LM were 21% (52/237) and by qRT-PCR 25% (107/426). 84% [CI_{95}: 79–88%] and 53% [CI_{95}: 43–63%] gametocytaemia was submicroscopic, for *Pv* and *Pf* respectively (Fig 1A and 1D). In one *Pv* and two *Pf* samples gametocytes were detected by LM but not by qRT-PCR, indicating most probably RNA degradation. For *Pv* infections, a late-stage trophozoite can be misread as a gametocyte, however LM slides of this study were read by three independent microscopists. Overall, gametocyte densities by LM and by molecular methods were significantly correlated in samples positive by both methods (Kendall's tau test, pvs25: tau = 0.24, *p*-value = 0.037, *pfs25*: tau = 0.23, *p*-value = 0.027). Due to the low sensitivity of LM in gametocyte detection, all further results presented here derive from molecular gametocyte detection.

Significantly more *Pv* infections carried qRT-PCR detectable gametocytes compared to *Pf* (38% vs. 25%, $p<0.001$). Microscopically patent infections of both species carried gametocytes more often than submicroscopic infections (*Pv*: 48% vs. 27%, *Pf*: 35% vs. 13%, $p<0.001$, Fig 1B and 1E). Similarly, gametocyte-specific transcript numbers were significantly higher in LM-positive than LM-negative samples for both species (S1 Fig).

Gametocyte positivity and density in symptomatic versus asymptomatic infections

During the follow-up period, 34 *Pv* episodes and 68 *Pf* clinical episodes were observed. The proportion of gametocyte carriers was 22% higher in clinical episodes compared to asymptomatic *P. vivax* infections (59% vs. 37%, $p = 0.014$, Fig 1C). For *P. falciparum*, a similar trend was observed but did not reach statistical significance (34% vs. 23%, $p = 0.098$, Fig 1F). However, due to a much higher number of asymptomatic infections than clinical episodes, the overwhelming majority of *Pv* and *Pf* gametocyte carriage (92% [CI_{95}: 88–95%] and 79% [CI_{95}: 69–86%]) occurred in asymptomatic children. *Pv* gametocyte densities showed the same trend as asexual densities in both clinical episodes and asymptomatic infections, but this was not the case for *Pf* (S2 Fig).

The effect of PQ treatment on gametocytaemia during follow-up

Pv gametocyte prevalence increased steadily throughout the follow-up period and was on average almost 3-fold higher in the placebo arm than in the PQ arm, similar to patterns observed in *Pv* blood-stage parasite prevalence (*Pv* gametocytes median fold difference PL>PQ: 2.9 [IQR: 2.0–3.8], *Pv* blood-stages median fold difference PL>PQ: 2.8 [IQR: 2.2–4.2], Fig 2A). No difference in *Pf* gametocyte prevalence was observed between study arms (*Pf* gametocytes median fold difference PL>PQ 1.0 [IQR: 0.7–1.4], *Pf* blood-stages median fold difference PL>PQ: 1.1 [IQR: 0.9–1.3], Fig 2B).

To assess in detail *Pv* gametocyte production in primary infections versus relapses, we compared gametocyte positivity and density in first infections after blood-stage plus placebo (first

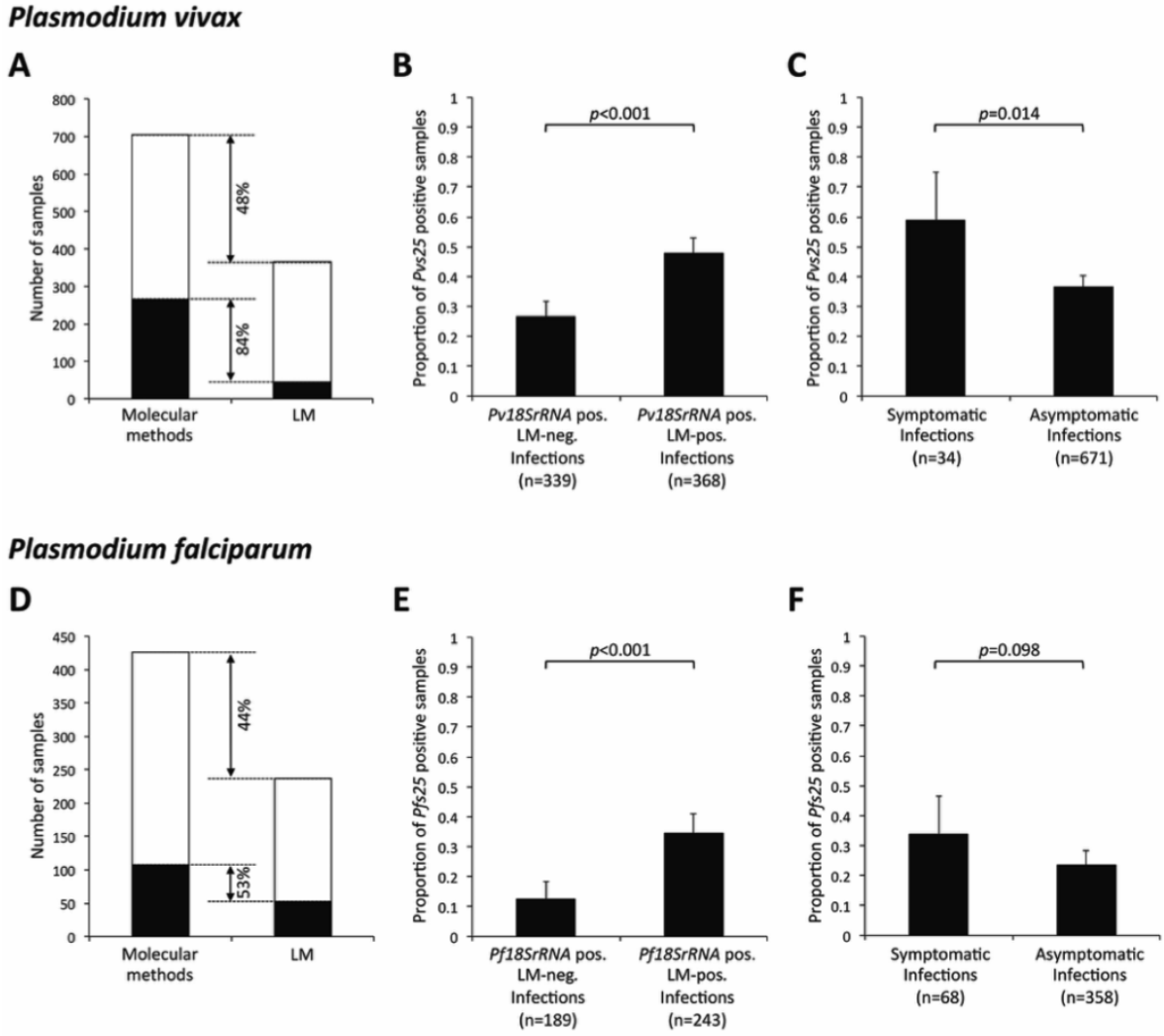

Fig 1. *Pv* (top) and *Pf* (bottom) gametocyte positivity among 5019 follow-up samples. (**A, D**) Detection of blood stage parasites and gametocytes by LM and molecular methods, using *pv18S* or *pf18S rRNA* qPCR for detection of blood-stage parasites and *pvs25* or *pfs25* qRT-PCR for detection of gametocytes. Black: gametocyte positive samples. White: parasite positive samples without gametocytes. (**B, E**) Proportion of gametocytes positives (by molecular methods) in submicroscopic and LM-positive samples. (**C, F**) Proportion of gametocyte positives (by molecular methods) in symptomatic and asymptomatic infections. Error bars indicate 95% confidence intervals by X^2 distribution.

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Pv infection either from relapse (80%80%) or infective bite (20%)) or blood-stage plus PQ treatment (first *Pv* infection always from infective bite) [15]. We also assessed subsequent *Pv* infections, i.e. all but the first parasite-positive sample per child, which in both arms can result from an ongoing infection, a relapsing hypnozoite or a new infection from a mosquito. First *Pv* re-infections after baseline treatment were equally likely to carry gametocytes in both

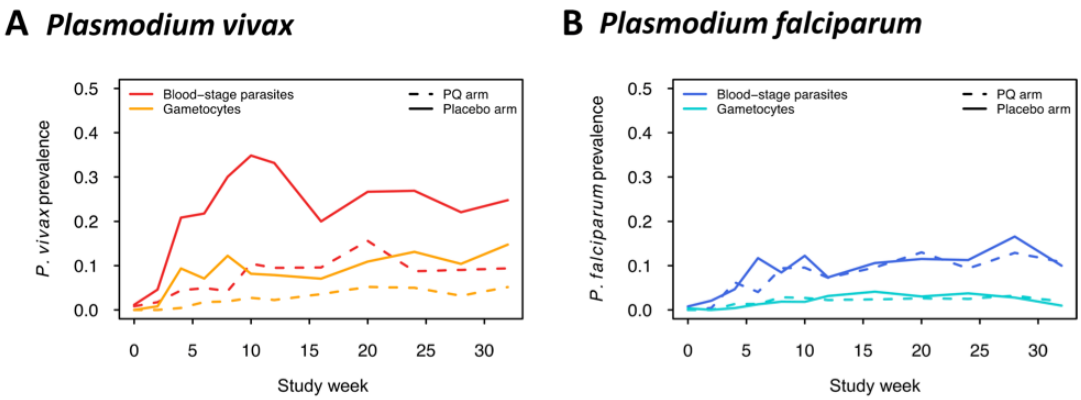

Fig 2. Prevalence of blood-stage parasites and gametocytes of *Pv* (A) and *Pf* (B) during follow-up by treatment arm.

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treatment arms (PQ: 29% vs. Placebo: 31%, Fig 3A), and the same was observed for subsequent infections (PQ: 42% [CI_{95}: 32–52%] vs. Placebo: 41% [CI_{95}: 36–47%], $p = 1$). To investigate whether gametocyte densities were simply following the asexual densities or if other factors play a role, we compared absolute as well as normalized gametocyte densities. Gametocyte densities were normalized by dividing *pvs25* or *pfs25* transcript numbers/μl by *Pv*- or *Pf-18S rRNA* copy numbers/μl. Absolute and normalized *Pv* gametocyte densities did not differ between treatment arms in first infections (Fig 3B and 3C) nor in subsequent infections (S3 Fig).

We also investigated *Pf* gametocyte carriage by comparing *Pf* gametocyte positivity and density in first *Pf* infections after treatment. Significantly more *Pf* gametocyte carriers were observed among first infections in the PQ-arm compared to the placebo arm (PQ: 26% vs. Placebo: 10%, Fig 3D). No significant difference between trial arms was observed in subsequent samples (PQ: 30% [CI_{95} 22–39%] vs. Placebo: 31% [CI_{95} 23–40%], p = 0.961). *Pf* absolute and normalized gametocyte densities did not differ significantly between the treatment arms (Fig 3E and 3F, S3 Fig).

Risk factors for gametocytes positivity and density

Pv gametocytes were detected more frequently (Table 1, OR for 1-log increase of density = 1.95, $p<0.001$) and in higher densities (Table 2, $p<0.001$) with increasing blood-stage parasite density. Apart from reducing the number of *Pv* positive samples during follow-up (S1 Table), PQ treatment had no further effect on *Pv* gametocyte positivity (Table 1).

In *Pv* positive samples, the odds of *Pv* gametocytes were 60% reduced and gametocyte densities were 30% lower in mixed *Pf*/*Pv* infections compared to single-species *Pv* infections (Table 1, $p<0.001$; Table 2 $p = 0.003$). The odds for *Pv* gametocyte carriage increased significantly over the whole follow-up period (Table 1 and Fig 2, $p<0.001$), and a 36% reduction on the odds of being gametocyte positive was observed in first *Pv* infections compared to subsequent infections (Table 1, $p = 0.040$). No other factors were associated with the odds for *Pv* gametocyte carriage during follow-up (Table 1). *Pv* gametocyte density, but not positivity, decreased with age (Table 2, $p = 0.017$) following the age trend in asexual parasites (Table 3, [29]).

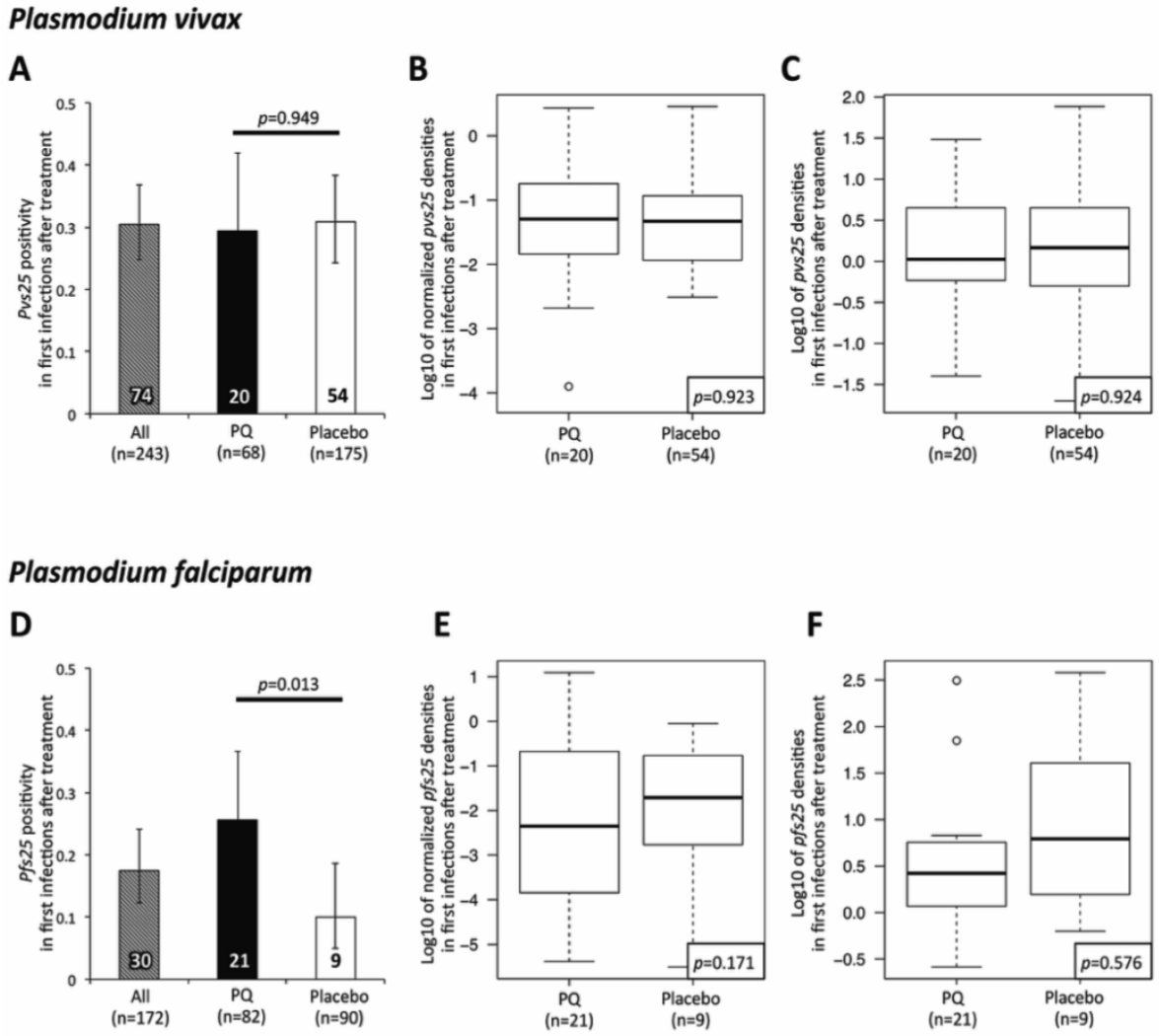

Fig 3. Gametocyte positivity and density in first *Pv* (top) and *Pf* (bottom) infections after treatment with blood-stage antimalarials alone (placebo) or blood-stage antimalarials plus PQ (PQ). (**A, D**). Proportion of *Pv* and *Pf* gametocyte carriers among first infections by treatment arm. Figures within the bars indicate absolute numbers of gametocyte-positive first infections following treatment. Error bars indicate 95% confidence intervals by X^2 distribution. (**B, E**). Normalized *Pv* and *Pf* gametocyte densities in first infections by treatment arm. Densities were normalized by dividing *pvs25* or *pfs25* transcript numbers/μl by *Pv*- or *Pf-18S rRNA* copy numbers/μl. (**C, F**) Absolute *Pv* and *Pf* gametocyte densities in first infections by treatment arm. Densities are expressed as $\log_{10}$ of *pvs25* and *pfs25* transcripts/μl.

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As for *Pv* gametocytes, the odds for *Pf* gametocytes were 70% reduced in mixed *Pf*/*Pv* infections compared to single-species *Pf* infections (Table 1, $p<0.001$). As an effect of delayed *Pf* gametocyte maturation, gametocyte positivity was 55% lower in first *Pf* infections compared to subsequent infections (Table 1, $p = 0.007$). Other risk factors for *Pf* gametocytes were investigated, but none of the parameters tested was significant. The *Pf* gametocyte positivity was

Table 1. Multivariable predictors of *Pv* and *Pf* gametocyte positivity during follow-up.

	Pv gametocyte positive				*Pf* gametocyte positive			
	OR	95% CI		*p*-value	OR	95% CI		*p*-value
Blood-stage density (by qPCR), per 10x increase	1.95	1.47	2.58	<0.001	1.23	0.99	1.51	0.059
PQ treatment	1.03	0.68	1.58	0.878	1.21	0.78	1.90	0.395
Mixed *Pf*/*Pv* (by qPCR)	0.39	0.25	0.62	<0.001	0.33	0.18	0.60	<0.001
First infection	0.64	0.42	0.98	0.040	0.45	0.25	0.81	0.007
Days after DOT (ref:0–60)								
61–120	0.59	0.39	0.89		1.31	0.62	2.76	
121–180	1.48	0.91	2.42	<0.001	1.32	0.63	2.75	0.266
>180	2.54	1.47	4.39		0.73	0.32	1.67	
Constant	0.36	0.22	0.59	<0.001	0.29	0.11	0.77	<0.013

OR, odds ratio

DOT, directly observed treatment.

ORs were obtained using binomial generalized estimating equations with logit-link allowing for repeated visits by back-selection from the full model. The full model included fever, infection status at enrolment by qPCR (*Pf* or *Pv* positive), LLIN use (less than 100%), sex, village of residence, hemoglobin at baseline (>9 g/dl), age. No significant interaction of PQ treatment with days post DOT was detected.

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slightly higher in samples with high asexual densities, yet this association did not reach the 5% significance level (Table 1, OR for 1-log increase of density = 1.23, $p = 0.059$). In contrast to *Pv*, *Pf* gametocyte densities were not associated with any of the factors assessed (S1 Text). Fever was strongly associated with increasing blood-stage *Pf* parasitaemia (Table 3, OR = 2.41, $p<0.001$), but had no effect on gametocyte density.

Analysis of only subsequent infections showed similar results to the analysis of the entire follow-up period (S2 Table). Considering subsequent infections only, *Pv* gametocytes were reduced by 47% [13–68%] and *Pf* gametocytes were reduced by 63% [26–82%] in mixed-species infections compared to single-species infections (*Pv* *p*-value: 0.013, *Pf* *p*-value: 0.006, S2 Table). It was not possible to analyse the effect of mixed-species co-infection on gametocyte carriage in first positive samples following treatment due to very low sample size for either species.

Table 2. Multivariable predictors of *Pv* and *Pf* gametocyte density during follow-up.

	Pv gametocyte density			
	exp(β)	95% CI		*p*-value
Blood-stage density (by qPCR) per 10x increase	1.37	1.20	1.56	<0.001
PQ treatment	0.97	0.80	1.18	0.765
Mixed *Pf*/*Pv* (by qPCR)	0.73	0.59	0.90	0.003
Age	0.94	0.89	0.99	0.017
Days after DOT (ref: 0–60)				
61–120	1.08	0.86	1.35	
121–180	1.15	0.90	1.47	0.163
>180	1.30	1.03	1.64	
Constant	1.11	0.69	1.81	0.661

β, regression coefficient.

Coefficients were obtained using Gaussian generalized estimating equations with log-link by allowing for repeated visits and by back-selection from the full model. The full model included fever, infection status at enrolment by qPCR (*Pf* or *Pv* positive), LLIN use (less than 100%), sex, village of residence, hemoglobin at baseline (>9 g/dl), first infection. No predictors were associated with *Pf* gametocyte densities (S1 Text). No significant interaction of PQ treatment with days post DOT was detected.

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Table 3. Multivariate predictors *Pv* and *Pf* blood-stage parasite density during follow-up.

	Pv blood-stage density				*Pf* blood-stage density			
	exp(β)	95% CI		*p*-value	exp(β)	95% CI		*p*-value
PQ treatment	1.04	0.93	1.15	0.505	0.85	0.68	1.06	0.143
Mixed *Pf*/*Pv* (by qPCR)	-	-	-	-	0.79	0.63	1.00	0.048
Fever	-	-	-	-	2.41	1.82	3.19	<0.001
Age	0.96	0.92	0.99	0.014	-	-	-	-
Days after DOT (ref: 0–60)								
61–120	0.94	0.83	1.07		0.93	0.69	1.25	
121–180	0.72	0.62	0.83	<0.001	0.89	0.62	1.25	<0.001
>180	0.51	0.44	0.59		0.59	0.43	0.80	
Constant	5.13	3.83	6.87	<0.001	16.37	12.37	21.66	<0.001

β, regression coefficient.

Coefficients were obtained using Gaussian generalized estimating equations with log-link by allowing for repeated visits and by back-selection from the full model. The full model included fever, infection status at enrolment by qPCR (*f.* or *v.* positive), LLIN use (less than 100%), sex, village of residence, hemoglobin at baseline (>9 g/dl), first infection. Non-associated predictors were shown by "-"in the respective line. No significant interaction of PQ treatment with days post DOT was detected.

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Discussion

This study represents a first detailed investigation of the contribution of *Pv* relapses to the infectious reservoir. The transmission potential attributable to relapses was estimated by comparing gametocyte positivity and density in children that had received either PQ or placebo treatment. A major finding was that *Pv* gametocytes were detected in equal proportions and equal density in *Pv* positive samples of both trial arms. In the PQ arm, the majority of *Pv* infections derived from new mosquito bites, while in the placebo arm 80% of infections were caused by relapsing hypnozoites [15]. Gametocyte densities as well as the proportion of gametocyte carriers concurred in both arms, thus indicating that new and relapsing infections produce gametocytes at equal rates. Similar conclusions were drawn from a study in south-east Asia, where *Pv* gametocyte densities and positivity had closely mirrored parasitaemia in both, clinical primary and recurrent infections [16]. Gametocyte production in relapses thus seems indistinguishable from that in new infections. This finding highlights the importance of anti-hypnozoite drugs to prevent relapses for an effective interruption of *Pv* transmission.

Sample storage in this cohort was not optimal for RNA preservation. Blood was spotted onto Whatman 3MM filter paper in the field, and stored at room temperature for up to 5 weeks until transferred into TRIzol reagent. This procedure was suboptimal compared to sampling in RNA-stabilizing reagents [19]. A more recent cross-sectional study in PNG employed sampling in RNAprotect Cell Reagent (Qiagen, Switzerland) and found gametocytes in 78% and 60% of *Pf* and *Pv* qPCR-positive samples in children aged 6–9 years [25]. Almost universal *Pv* gametocyte prevalence (95%) was found in Brazilian samples stored in liquid nitrogen [30]. The relatively low gametocyte positivity in this cohort was indicative of poor RNA quality, which likely resulted in a substantial underestimation of gametocyte rates. The gametocyte rate in the present study thus reflects a minimum prevalence. Because RNA quality and sample volume did not vary within the study, the comparative analyses of treatment arms and risk factors remain unaffected, even if these results need to be regarded as referring to infections with moderately high gametocyte densities.

The vast majority (>80%) of gametocyte carriers were asymptomatic for both species, and over 20% of *Pv* and over 30% of *Pf* gametocyte positive samples were submicroscopic.

Although gametocyte densities were lower in submicroscopic infections compared to LM-positive infections for both species, they may nonetheless be potentially infective to mosquitoes. Mosquito feeding experiments have demonstrated that submicroscopic infections can infect mosquitoes, albeit at lower rates than microscopically patent infections, and thus contribute to onward transmission [31–35]. Our results highlight the importance of treating all malaria infections in the community, as asymptomatic individuals will not report themselves to health facilities and thus generally remain untreated and infectious for longer periods.

Co-infections with both species are common in PNG [36,37] including in this cohort, and interactions between co-infecting species in mixed infections have been investigated previously. However, these former studies focused on the asexual stages of *Pv* and *Pf* [38,39] or risk for clinical diseases [29,38,40] and did not address transmission stages. Gametocytes in the host are influenced by a complex interplay of parasite factors (such as stress response) and host factors (such as immunity), and this complexity is enhanced by a second co-infecting *Plasmodium* species. Our finding of significantly reduced gametocytes in mixed-species infections compared to single-species infections is a first indication of species interaction affecting the transmission stages. Confirmation of our results is required in other studies investigating *Plasmodium* species interactions with specific focus on the transmission stages.

In the first post-treatment *Pf* infections gametocytes were more frequently detected in the PQ arm than in the placebo arm. This is likely explained by the slower acquisition of new *Pv* infections in PQ-treated individuals, compared to a fast relapse rate in individuals retaining hypnozoites in the liver. Indeed, in the placebo arm 52% of first *Pf* infections carried a *Pv* co-infection as opposed to only 21% in the PQ arm. Accordingly, the multivariate analysis showed reduced odds of *Pf* as well as *Pv* gametocytes in mixed-species infections compared to single-species infections. In addition, a *Pf* co-infection reduced *Pv* gametocyte densities by half. A study in 0.5 to 5 year old PNG children with uncomplicated malaria confirmed that *Pv* gametocytaemia in *Pf/Pv* mixed infections was reduced compared to *Pv* single infections [41]. Moreover, a community study in PNG showed a lower proportion of *Pf* gametocyte carriers in *Pf/Pv* mixed infections compared to *Pf* single-species infections [25]. Similar findings had been reported from Thailand [42]. More longitudinal studies designed specifically to address gametocyte dynamics in mono- and mixed-species infections are needed to confirm potential cross-species interaction and its effect on sexual stage development.

Conclusion

Onset and rate of *Pv* gametocyte production did not differ between relapses and primary infections. This is a strong argument for treatment policies and elimination strategies that support PQ treatment of all *Pv* infections. The vast majority of gametocyte carriers in this study were detected in asymptomatic infections, which suggests that sensitive detection and early treatment of asymptomatic and submicroscopic Plasmodium *spp*. infections may be crucial for an effective control of transmission. PQ treatment prevented relapses and thus reduced *Pv* gametocyte carriage by 73%. These and other *Plasmodium* species interactions that can substantially affect gametocyte production warrant further investigation.

Supporting information

S1 Text. Evaluation of the multivariate analysis (GEE models) of *P. vivax* and *P. falciparum*.
(DOCX)

S1 Fig. Gametocyte and overall parasite density in submicroscopic and LM-positive *Pv* (top) and *Pf* (bottom) infections. A. and C. *Pv* and *Pf* gametocyte densities were expressed as log10 of *pvs25* and *pfs25* transcripts/μl. B. and D. *Pv* and *Pf* parasite densities were expressed as log10 of *pv18S rRNA* and *pf18S rRNA* gene copies/μl. C. and E. *Pv* and *Pf* normalized gametocyte densities. Densities were normalized by division of *pvs25* and *pfs25* transcripts/μl by *pv18S rRNA* or *Pf18S rRNA* genomic copies/μl, respectively.
(DOCX)

S2 Fig. Gametocyte and parasite density in symptomatic and asymptomatic *Pv* (top) and *Pf* (bottom) infections. A. and C. *Pv* and *Pf* gametocyte densities were expressed as log10 of *pvs25* and *pfs25* transcripts/μl. B. and D. *Pv* and *Pf* parasite densities were expressed as log10 of *pv18S rRNA* and *pf18S rRNA* gene copies/μl. C. and E. *Pv* and *Pf* normalized gametocyte densities. Densities were normalized by division of *pvs25* and *pfs25* transcripts/μl by *pv18S rRNA* or *Pf18S rRNA* genomic copies/μl, respectively.
(DOCX)

S3 Fig. Gametocyte positivity and density in subsequent (i.e. not first) *P. vivax* (top) and *P. falciparum* (bottom) infections after treatment with blood-stage antimalarials alone (Placebo) or blood-stage antimalarials plus Primaquine (PQ). A. and C. Proportion of *P. vivax* and *P. falciparum* gametocyte carriers among subsequent infections by treatment arm. Figures within the bars indicate absolute numbers of gametocyte-positive subsequent infections following treatment. Error bars indicate 95% confidence intervals by X^2 distribution. B. and E. Normalized *P. vivax* and *P. falciparum* gametocyte densities in subsequent infections by treatment arm. Normalization was done by dividing *pvs25* or *pfs25* transcript numbers/μl by *Pv-* or *Pf-18S rRNA* copy numbers/μl. C. and F. Absolute *P. vivax* and *P. falciparum* gametocyte densities in subsequent infections by treatment arm. Densities are expressed as $\log_{10}$ of *pvs25* and *pfs25* transcripts/μl.
(DOCX)

S1 Table. Multivariate risk factors of *P. vivax* and *P. falciparum* asexual parasite positivity during follow-up.
(DOCX)

S2 Table. Multivariate risk factors of *P. vivax* and *P. falciparum* gametocyte carriage in subsequent infections during follow-up.
(DOCX)

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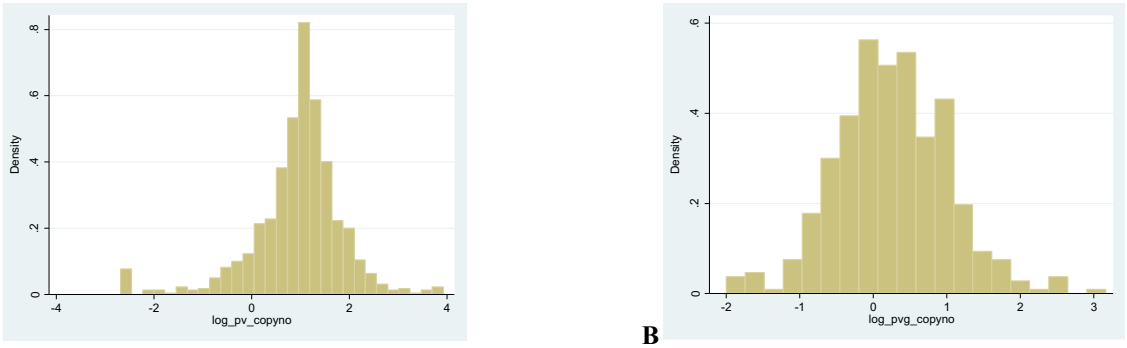
SUPPLEMENT

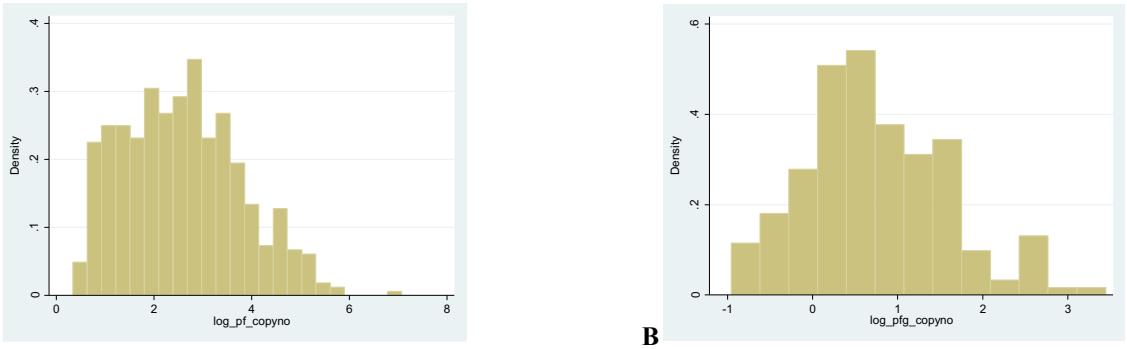
S1 TEXT

Evaluation of the multivariate analysis (GEE models) of *P. vivax* and *P. falciparum.*



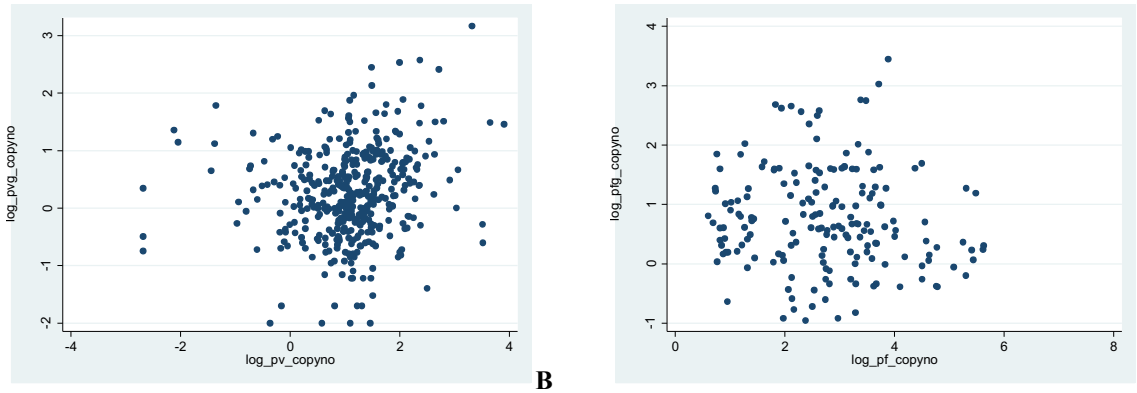
A B

Figure S1.1 Density distribution of *P. vivax*. (A) *Pv* blood-stage densities by genomic 18S rRNA copies/µl (log_{10}). (B) *Pv* gametocytes densities by *pvs25* transcript copies/µl (log_{10}). Log_{10} transformations of copies/µl show normal distribution.



A B

Figure S1.2 Density distribution of *P. facliparum*. (A) *Pf* blood-stage densities by genomic 18S rRNA copies/µl (log_{10}). (B) *Pf* gametocytes densities by *pfs25* transcript copies/µl (log_{10}). Log_{10} transformations of copies/µl show almost normal distribution.



A B

Figure S1.3 Scatterplot of *Pv* (A) and *Pf* (B) blood-stage vs. gametocyte density (log_{10}).

Supplementary Figure S1

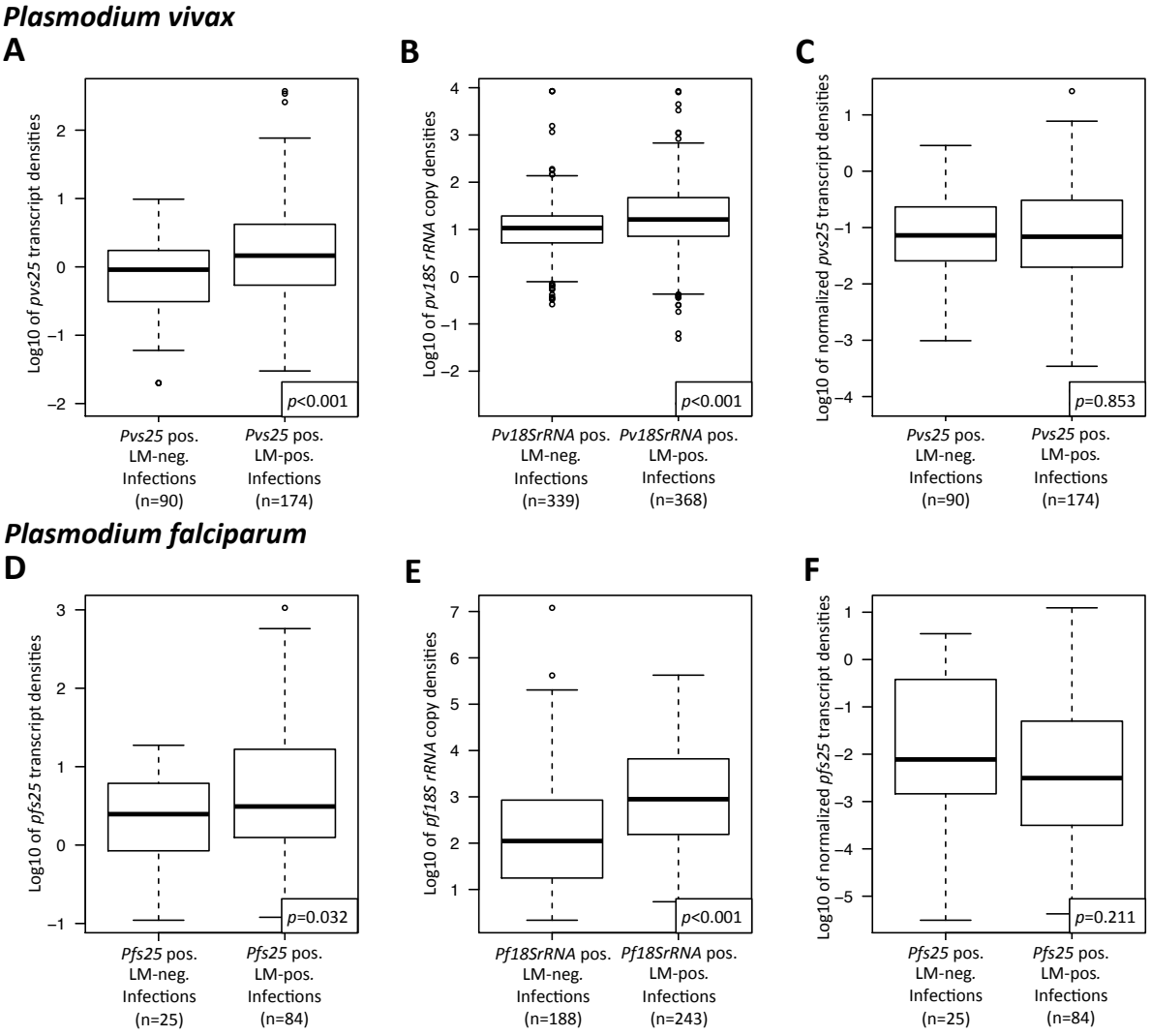


Figure S1. Gametocyte and overall parasite density in submicroscopic and LM-positive *Pv* (top) and *Pf* (bottom) infections. A. and C. *Pv* and *Pf* gametocyte densities were expressed as $\log_{10}$ of *pvs25* and *pfs25* transcripts/µl. B. and D. *Pv* and *Pf* parasite densities were expressed as $\log_{10}$ of *Pv*- and *Pf-18S rRNA* gene copies/µl. C. and E. *Pv* and *Pf* normalized gametocyte densities. Densities were normalized by division of *pvs25* and *pfs25* transcripts/µl by *Pv*- or *Pf-18S rRNA* genomic copies/µl, respectively.

Supplementary Figure S2

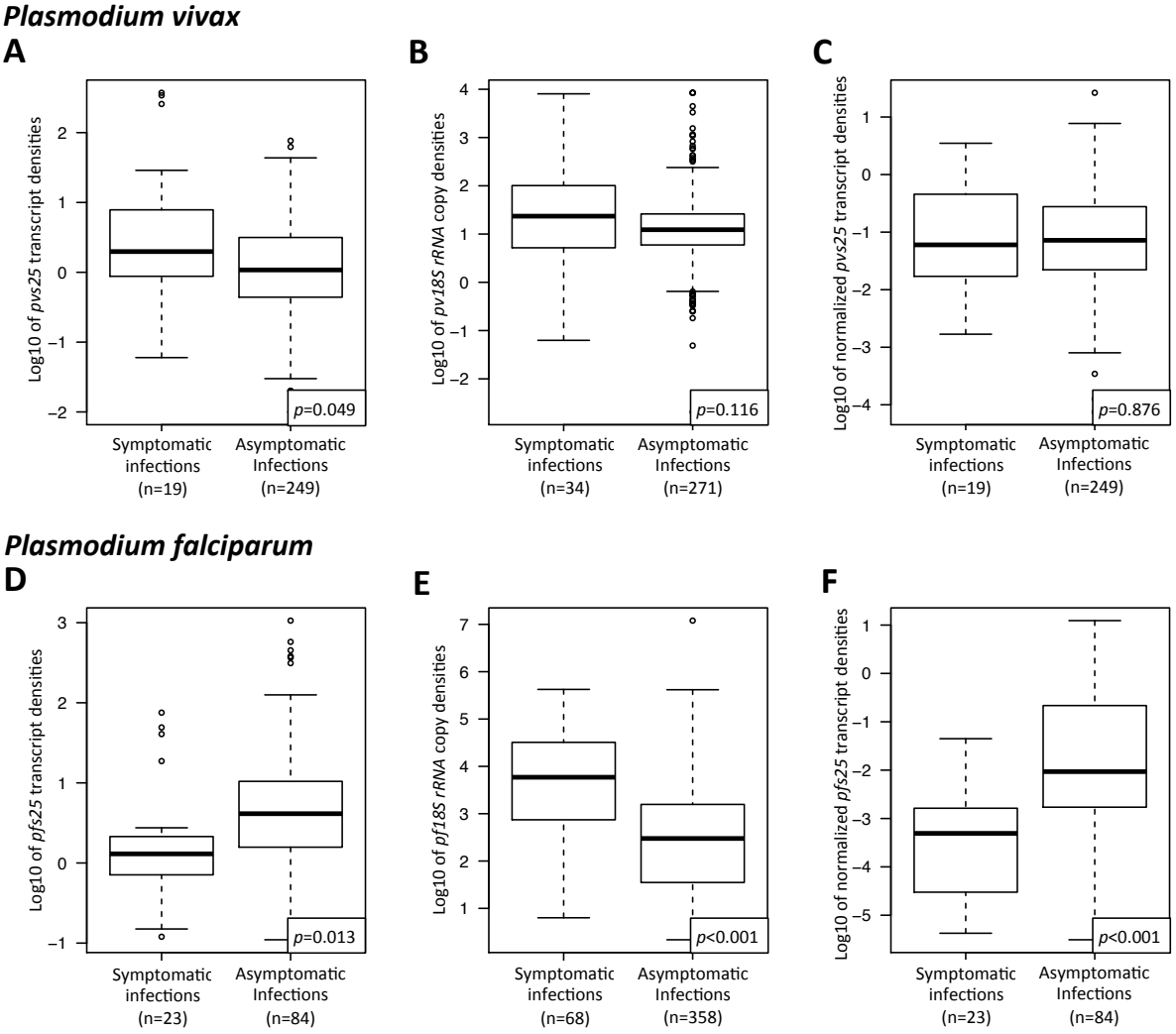


Figure S2. Gametocyte and parasite density in symptomatic and asymptomatic *Pv* (top) and *Pf* (bottom) infections. A. and C. *Pv* and *Pf* gametocyte densities were expressed as $\log_{10}$ of *pvs25* and *pfs25* transcripts/µl. B. and D. *Pv* and *Pf* parasite densities were expressed as $\log_{10}$ of *Pv*- and *Pf-18S rRNA* gene copies/µl. C. and E. *Pv* and *Pf* normalized gametocyte densities. Densities were normalized by division of *pvs25* and *pfs25* transcripts/µl by *Pv*- or *Pf-18S rRNA* genomic copies/µl, respectively.

Supplementary Figure S3

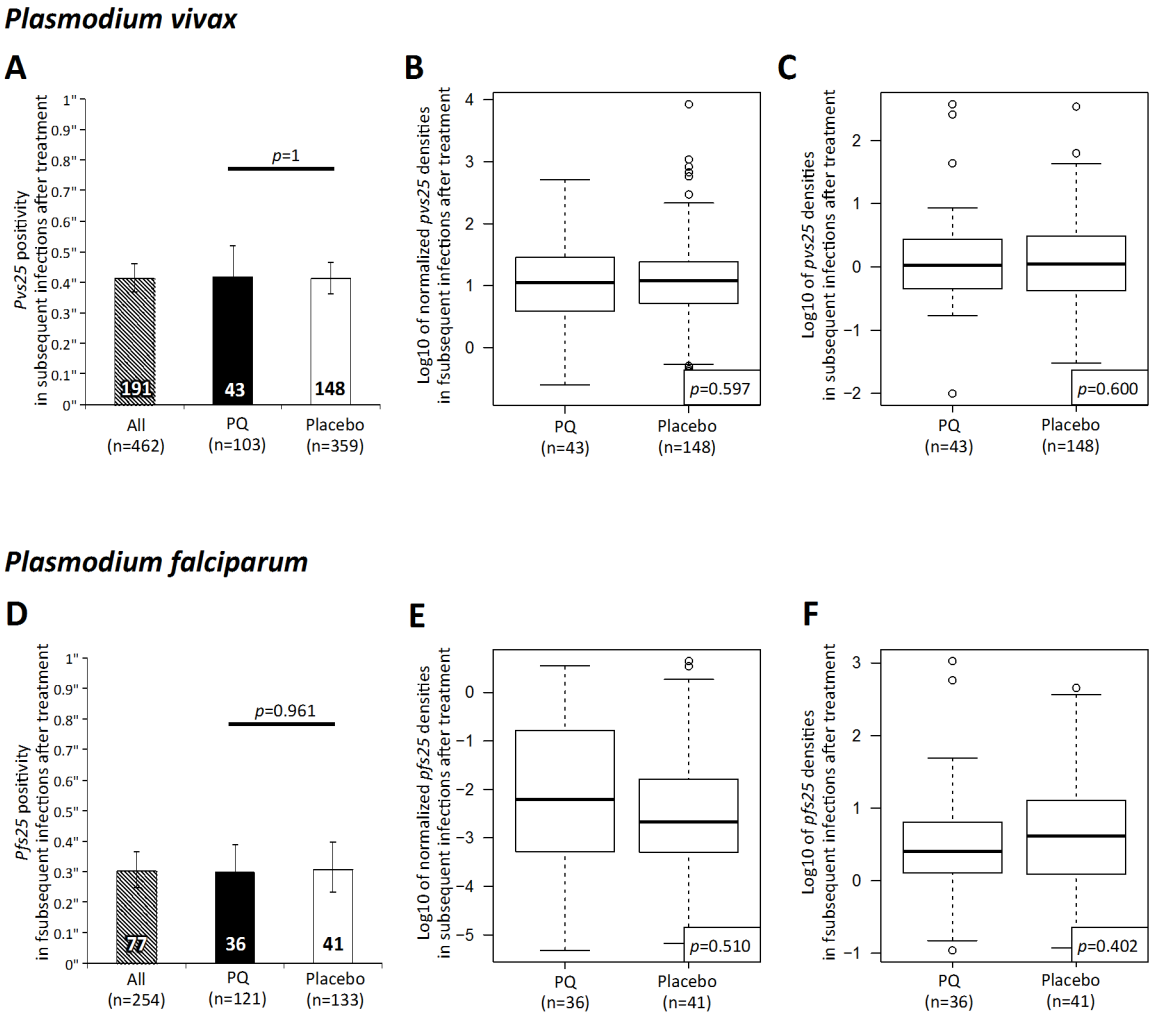


Figure S3. Gametocyte positivity and density in subsequent (i.e. not first) *Pv* (top) and *Pf* (bottom) infections after treatment with blood-stage anti-malarials alone (Placebo) or blood-stage anti-malarials plus Primaquine (PQ). A. and C. Proportion of *Pv* and *Pf* gametocyte carriers among subsequent infections by treatment arm. Figures within the bars indicate absolute numbers of gametocyte-positive subsequent infections following treatment. Error bars indicate 95% confidence intervals by Chi^2 distribution. B. and E. Normalized *Pv* and *Pf* gametocyte densities in subsequent infections by treatment arm. Normalization was done by dividing *pvs25* or *pfs25* transcript numbers/µl by *Pv*- or *Pf-18S rRNA* copy numbers/µl. C. and F. Absolute *Pv* and *Pf* gametocyte densities in subsequent infections by treatment arm. Densities are expressed as log_{10} of *pvs25* and *pfs25* transcripts/µl.

Chapter 6:

Template competition in PCR of length-polymorphic markers

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EPIDEMIOLOGY AND SURVEILLANCE

Critical Evaluation of Molecular Monitoring in Malaria Drug Efficacy Trials and Pitfalls of Length-Polymorphic Markers

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ABSTRACT Estimation of drug efficacy in antimalarial drug trials requires parasite genotyping to distinguish new infections from treatment failures. When using length-polymorphic molecular markers, preferential amplification of short fragments can compromise detection of coinfections, potentially leading to misclassification of treatment outcome. We quantified minority clone detectability and competition among *msp1*, *msp2*, and *glurp* amplicons using mixtures of *Plasmodium falciparum* strains and investigated the impact of template competition on genotyping outcomes in 44 paired field samples. Substantial amplification bias was detected for all three markers, with shorter fragments outperforming larger fragments. The strongest template competition was observed for the marker *glurp*. Detection of *glurp* fragments in multiclonal infections was severely compromised. Eight of 44 sample pairs were identified as new infections by all three markers. Ten pairs were defined as new infections based on one marker alone, seven of which were defined by the questionable marker *glurp*. The impact of size-dependent template competition on genotyping outcomes therefore calls for necessary amendments to the current WHO recommendations for PCR correction of malaria drug trial endpoints. Accuracy of genotyping outcomes could be improved by separate amplification reactions per allelic family and basing results on markers *msp1* and *msp2* first, with *glurp* only used to resolve discordant results.

KEYWORDS genotyping, PCR, *Plasmodium falciparum*, amplification bias, drug trial, *glurp*, *msp1*, *msp2*, template competition

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In areas of high malaria endemicity, individuals are often multiply infected with *Plasmodium falciparum*. The number of concurrent clones per infected individual is denoted the multiplicity of infection (MOI). The MOI can reach a mean of five clones per infection in high-transmission areas and usually ranges between one and two clones per infected individual in regions of intermediate or low transmission (1). Concurrent clones in multiclone infections are distinguished by genotyping of polymorphic molecular markers. In *in vivo* efficacy drug or vaccine trials in settings where malaria is endemic, parasite genotyping for discrimination of newly incoming versus recrudescent clones after antimalarial treatment is essential for accurate estimation of the efficacy of the intervention (2, 3). Genotyping is further used in malaria molecular epidemiology to assess MOI as a proxy of transmission level in molecular monitoring of interventions and for tracking of individual clones over time in cohort studies to measure the molecular force of infection or duration of infection (4, 5).

The most commonly used *P. falciparum* molecular markers for genotyping are the length-polymorphic genes encoding the merozoite surface proteins 1 and 2 (*msp1* and *msp2*) and the glutamate-rich protein (*glurp*) (6, 7). The adoption of capillary electrophoresis (CE)-based sizing of PCR products largely resolved limitations of gel-based

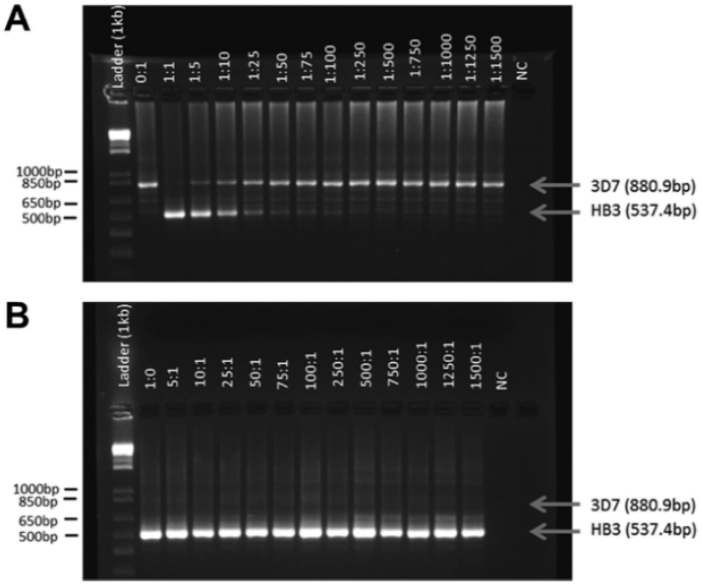

FIG 1 Agarose gel of *glurp* nPCR products obtained from mixtures of *P. falciparum in vitro* culture strains HB3 and 3D7 in different ratios. (A) Strain 3D7 is predominant. (B) Strain HB3 is predominant. NC, negative control. Arrows indicate the sizes of PCR fragments as measured by CE.

sizing protocols, such as insufficient resolution of minimal size differences, particularly among fragments larger than 500 bp, or unequal loading of variable PCR yields leading to apparent but artificial size differences. These limitations are particularly relevant for *glurp* genotyping, since amplicons of the *glurp* R2 region often surpass 1,000 bp in size, and CE-based *glurp* genotyping was not routinely used in most laboratories (8, 9).

Parasite clones occasionally remain undetected by PCR despite persisting in a host, an observation described as imperfect clone detectability. The detectability of a clone in an individual sample has been estimated at around 60% in young children from Papua New Guinea (10) and Ghana (5, 11–14) and was found to decrease to 20% in Ghanaian adults older than 60 years (5, 11–14). Imperfect detectability is often attributed to periodical sequestration or fluctuations of parasite densities around the detection limit of PCR as a consequence of naturally acquired immunity to variant antigens. How much technical reasons contribute to imperfect detectability of individual clones has not been investigated systematically for the three prime *P. falciparum* genotyping markers *msp1*, *msp2*, and *glurp*, but circumstantial observations of clone competition during PCR can be found in the literature (15, 16).

Template competition among differently sized amplicons during PCR as well as the presence of a predominant clone might limit detectability of minority clones in multiclonal infections. This study thus aimed to improve the accuracy of the recommended genotyping procedures for *P. falciparum* typing routinely applied in malaria drug trials (17) and to establish the dimension of technical limitations in genotyping of recurrent parasitemias. The present findings indicate a dramatic effect of competition during PCR in favor of the smallest fragment.

RESULTS

***glurp* genotyping.** We observed significant preferential amplification of the shorter fragment in mixtures of two *P. falciparum* laboratory strains, 3D7 and HB3, carrying different *glurp* alleles. For the 1:1 ratio, only the shorter HB3 amplicon 537 bp in size was visible after fragment separation on agarose gel (Fig. 1A). The longer 3D7 fragment

TABLE 1 *msp1* and *msp2* allelic families and allele sizes (rounded mean fragment sizes determined by capillary electrophoresis) of the selected *P. falciparum in vitro* culture strains

Strain	Allelic family (allele size [bp])	
	msp1[a]	*msp2*
HB3	Mad20 type (158)	Fc27 type (337)
3D7	K1 type (248)	3D7 type (265)
K1	K1 type (177)	Fc27 type (407)
FCB1	Mad20 type (194)	3D7 type (342)

[a]The third *msp1* allelic family, RO33 type, is not length polymorphic and was not included in the analysis of experimental mixtures of culture strains.

(881 bp) was only detected if present at least 5-fold in excess over the shorter fragment (Fig. 1A). The HB3 minority clone demonstrated stronger bands than the 3D7 dominant clone up to a HB3/3D7 ratio of 1:10. In the reciprocal dilution series with HB3 as the dominant clone, amplification of the longer 3D7 fragment was completely suppressed by the shorter HB3 amplicon (Fig. 1B). The accuracy and precision of *glurp* sizing were substantially improved by CE compared to agarose gel, but due to the high fluorescence cutoff required to account for characteristic high stutter peaks in *glurp* PCR-CE, the detectability of minority clones was not increased.

***msp1* and *msp2* genotyping.** Unlike *glurp*, the alleles of markers *msp1* and *msp2* fall into three and two allelic families, respectively (Table 1). When family-specific *msp1* or *msp2* nested PCRs (nPCRs) were initially multiplexed, pronounced competition between templates was observed irrespective of whether clones belonged to the same or different allelic families. For example, in *msp2* PCR using mixtures of 3D7 (3D7 type) and HB3 (Fc27 type), the minority clone was only detected in ratios up 1:250. To avert such template competition, at least for allele mixtures of different families, all *msp1* and *msp2* nPCRs were performed as simplex family-specific reactions. This strategy increased clone detectability in mixtures of alleles from alternative families drastically, so that the minority clone was detected in all ratios up to 5,000:1 for markers *msp1* and *msp2* (Fig. 2A and B, right panels). However, template competition will still persist if alleles of the same family occur together.

When two mixed strains carried *msp1* or *msp2* alleles from the same allelic family, the shorter fragment was always preferentially amplified in 1:1 ratios (Fig. 3). The extent of template competition between alleles of the same family was quantified in a systematic analysis of different ratios of two culture strains, which showed that amplicon size had a dramatic effect on PCR outcome (Fig. 2). In mixtures of strains K1 and 3D7, which harbor *msp1* alleles of the same allelic family, the small *msp1* K1 allele (177 bp) was still detected in a 500-fold underrepresentation. In the reciprocal dilution with K1 as the dominant clone, the longer 3D7 allele (248 bp) was only detectable up to a K1/3D7 dilution ratio of 5:1 (Fig. 2A, left panel). A similar picture was observed for the Mad20-type *msp1* family: the shorter HB3 allele was detectable even if 200-fold underrepresented, but vice versa, the longer FCB1 allele was only detectable if it was no more than 5-fold underrepresented (Fig. 2A, middle panel). Results for marker *msp2* mirrored findings from *msp1* in that the shorter allele was preferentially amplified in PCR (Fig. 2B).

CE peak heights did not reflect the ratio of strains mixed (Fig. 3; see Fig. S1 in the supplemental material). The proportions of fluorescent signal from the minority versus dominant clone (Fig. S1), as well as the relative detection limit of a minority clone (Fig. 2), differed between markers and allelic families. *msp2* fragment lengths as well as the size differences between *msp2* alleles were larger than those of *msp1*, and coherently, the detection limit of *msp2* minority clones was reached at lower ratios than *msp1* genotyping (Fig. 2). Not only the size difference between amplicons but also overall PCR fragment size may thus contribute to the observed amplification bias.

***glurp* clone detectability in mixtures of four culture strains.** Complex ratios of four culture strains were also assessed. In a 1:1:1:1 mixture of strains, *glurp* fluorescent

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A

msp1

Culture strains	Both alleles of K1-type/ Same tube reactions			Both alleles of K1-type/ Same tube reactions			Both alleles of Mad20-type/ Same tube reactions			Both alleles of Mad20-type/ Same tube reactions			Mixed families/ Separate tube reactions			Mixed families/ Separate tube reactions		
	K1 177 bp	:	3D7 248 bp	K1 177 bp	:	3D7 248 bp	HB3 158 bp	:	FCB1 194 bp	HB3 158 bp	:	FCB1 194 bp	HB3 158 bp	:	3D7 248 bp	HB3 158 bp	:	3D7 248 bp
Ratios in mixture	1	:	1	1	:	1	1	:	1	1	:	1	1	:	1	1	:	1
	5	:	1	1	:	5	5	:	1	1	:	5	5	:	1	1	:	5
	25	:	1	1	:	25	25	:	1	1	:	25	25	:	1	1	:	25
	50	:	1	1	:	50	50	:	1	1	:	50	50	:	1	1	:	50
	75	:	1	1	:	75	75	:	1	1	:	75	75	:	1	1	:	75
	100	:	1	1	:	100	100	:	1	1	:	100	100	:	1	1	:	100
	200	:	1	1	:	200	200	:	1	1	:	200	250	:	1	1	:	250
	300	:	1	1	:	300	300	:	1	1	:	300	500	:	1	1	:	500
	400	:	1	1	:	400	400	:	1	1	:	400	750	:	1	1	:	750
	500	:	1	1	:	500	500	:	1	1	:	500	1000	:	1	1	:	1000
	1000	:	1	1	:	1000	1000	:	1	1	:	1000	5000	:	1	1	:	5000

K1-like alleles Mad20-like alleles

B

msp2

Culture strains	Both alleles of 3D7-type/ Same tube reactions			Both alleles of 3D7-type/ Same tube reactions			Both alleles of FC27-type/ Same tube reactions			Both alleles of FC27-type/ Same tube reactions			Mixed families/ Separate tube reactions			Mixed families/ Separate tube reactions		
	3D7 265 bp	:	FCB1 342 bp	3D7 265 bp	:	FCB1 342 bp	HB3 337 bp	:	K1 407 bp	HB3 337 bp	:	K1 407 bp	3D7 265 bp	:	HB3 337 bp	3D7 265 bp	:	HB3 337 bp
Ratios in mixture	1	:	1	1	:	1	1	:	1	1	:	1	1	:	1	1	:	1
	5	:	1	1	:	5	5	:	1	1	:	5	5	:	1	1	:	5
	25	:	1	1	:	25	25	:	1	1	:	25	25	:	1	1	:	25
	50	:	1	1	:	50	50	:	1	1	:	50	50	:	1	1	:	50
	75	:	1	1	:	75	75	:	1	1	:	75	75	:	1	1	:	75
	100	:	1	1	:	100	100	:	1	1	:	100	100	:	1	1	:	100
	200	:	1	1	:	200	200	:	1	1	:	200	250	:	1	1	:	250
	300	:	1	1	:	300	300	:	1	1	:	300	500	:	1	1	:	500
	400	:	1	1	:	400	400	:	1	1	:	400	750	:	1	1	:	750
	500	:	1	1	:	500	500	:	1	1	:	500	1000	:	1	1	:	1000
	1000	:	1	1	:	1000	1000	:	1	1	:	1000	5000	:	1	1	:	5000

3D7-like alleles FC27-like alleles

FIG 2 Limit of detection of *msp1* (A) and *msp2* (B) minority clones in mixtures of *P. falciparum* culture strains. The limit of detection was determined by reciprocal serial dilution of the minority clone. Culture strains carried alleles either of the same allelic family (same tube amplification, left and middle panels) or of different families (amplification in separate tubes, right panels). Green square, allele detected in CE; gray square, allele not detected in CE. Fragments in bp reflect rounded mean allele sizes determined by capillary electrophoresis.

signal and thus the relative proportion of amplified fragments decreased with increasing amplicon size (Fig. 4A). A 2-fold overrepresentation of the clone with the shortest *glurp* allele (HB3, 537 bp) led to complete suppression of all other fragments after applying the peak height cutoff (Fig. 4B, left panel). In contrast, when strain 3D7 (carrying the longest *glurp* fragment) increased in abundance, it remained undetected

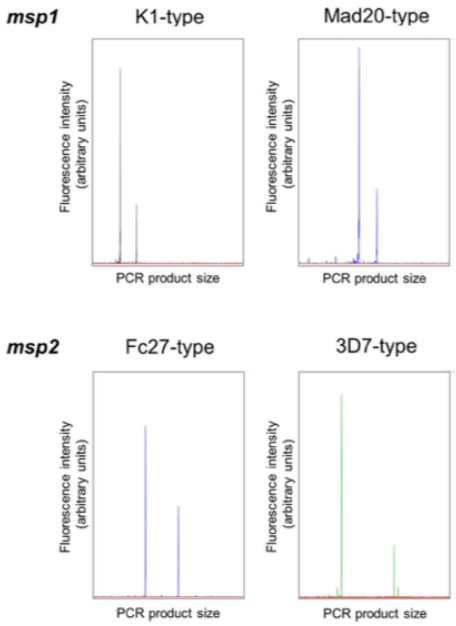

FIG 3 Electropherogram of *msp1* and *msp2* alleles from the same allelic family, amplified from culture strains mixed at a 1:1 ratio.

until in 5-fold excess over the other clones (Fig. 4B, right panel). The two shortest clones were successively lost only when 25-fold and 50-fold underrepresented toward 3D7 (Fig. 4B, right panel). Intermediate results were obtained when K1 and FCB1 were dominant within the 4-culture strain ratios (see Fig. S2 in the supplemental material).

The extent of template competition between several concurrent clones in *msp1* and *msp2* PCR depends largely on the composition of allelic families in the mixture. For a reliable assessment of amplification bias among more than two alleles per family of *msp1* and *msp2*, a large number of different culture strains would be required.

Genotyping of field samples. Marker performance was compared in genotyping of recurrent parasitemias using 44 paired pre- and posttreatment field samples. The mean *msp2* MOI was slightly higher after simplex allelic family-specific nPCR compared to duplex nPCR (2.2 versus 2.0 clones/infection). With a mean MOI of 1.4 clones per infection, *glurp* consistently identified fewer coinfecting clones compared to markers *msp1* and *msp2* (simplex nPCRs; mean *msp1* and *msp2* MOI of 2.2 clones/infection). Particularly in samples with a high *msp1* or *msp2* MOI, minority clone detection by marker *glurp* was severely limited (Fig. 5A). The mean number of clones detected did not differ between recrudescences and new infections for any marker (Fig. 5B; Wilcoxon *P* value of >0.05).

Genotyping outcomes of all three markers, *msp1*, *msp2*, and *glurp*, agreed for 30/44 (68%) of sample pairs, whereby 22 clear recrudescences and 8 clear new infections were identified (Table 2). When applying the current WHO-recommended approach of typing markers sequentially, all remaining 14 infections were characterized as new infections (Table 2). However, for 7/14 sample pairs, this classification was only supported by questionable marker *glurp*, while both *msp1* and *msp2* denoted a recrudescence. This suggests false classification due to imperfect *glurp* minority clone detectability using the current classification approach. A more appropriate classification would be based on the two better-performing markers *msp1* and *msp2*, which would diagnose these 7 pairs as recrudescence. For the remaining 7/14 sample pairs, markers *msp1* and *msp2*

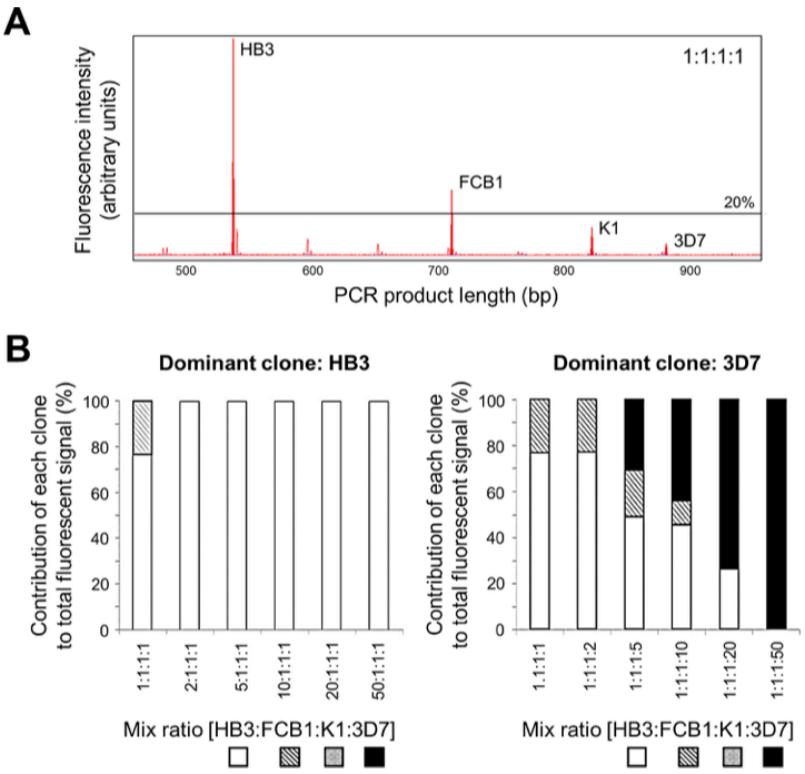

FIG 4 *glurp* minority clone detectability in mixtures of four culture strains. (A) Electropherograms of *glurp* alleles of four *P. falciparum* culture strains (HB3, FCB1, K1, and 3D7) mixed at a 1:1:1:1 ratio. (B) Proportion of *glurp* fluorescent signal detected during capillary electrophoresis for each clone in a four-culture strain mixture, with clone HB3 (shortest *glurp* allele, left panel) or clone 3D7 (longest *glurp* allele, right panel) as the increasingly dominant clone.

gave disparate results. Two approaches were tested for final classification of these seven sample pairs: first, our proposed approach 1 is based on support from two out of three markers *msp1*, *msp2*, and *glurp*; second, our proposed approach 2 is based on markers *msp1* and *msp2* only, but defining as a decisive characteristic for a new infection a complete switch in allelic family between the pre- and posttreatment samples. In other words, a sample pair was only characterized as a new infection if all alleles detected in the posttreatment sample were different from those in the pretreatment sample and belonged to an allelic family not present in the pretreatment sample. These approaches classified 3/7 (approach 1) or 4/7 (approach 2) sample pairs as recrudescence (see Table S3 in the supplemental material).

In total, 75% (32/44 using approach 1 and 33/44 using approach 2) of recurrent parasitemias after treatment were defined as recrudescent when using the proposed approaches for classification (Table 2). In contrast, only 50% (22/44) would be defined as recrudescent when one marker alone (in 7/14 cases, this was marker *glurp*) was permitted to define a new infection. This suggests misclassification of a third of all recrudescences as new infections by the current approach, mainly due to the marker *glurp*.

DISCUSSION

The present study allowed investigation of the impact of PCR amplification bias in a controlled environment, revealing a bias more severe than anticipated. A great

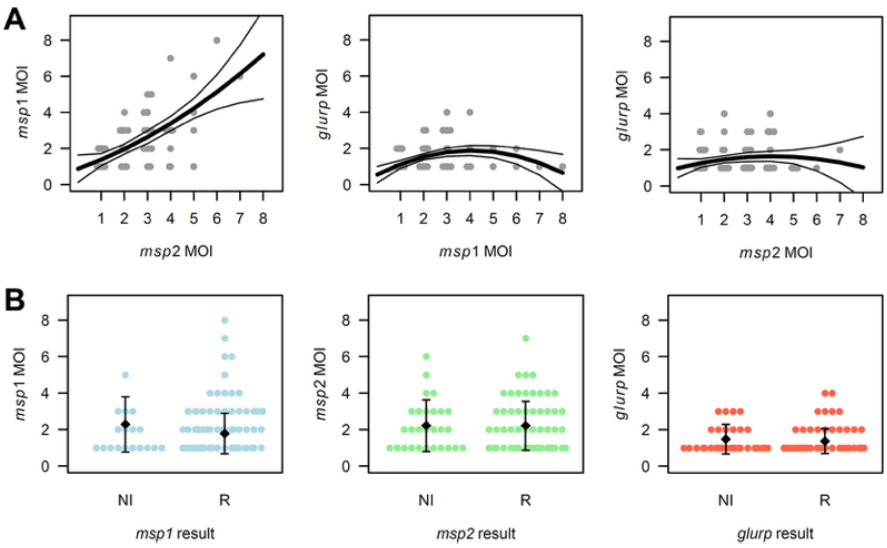

FIG 5 Patterns in *msp1*, *msp2*, and *glurp* MOI in 44 paired pre- and posttreatment samples. (A) Correlation of *msp2*/*msp1*, *glurp*/*msp1*, and *glurp*/*msp2* MOI per sample. Correlations were fitted using 2nd-degree polynomials (thick line) and are shown with 95% confidence intervals (thin lines). (B) *msp1*, *msp2*, and *glurp* MOI per sample classified by genotyping outcome. NI, new infection; R, recrudescence. Mean MOI per class and standard deviation are shown.

advantage of shorter over longer fragments was confirmed, with the strongest effect observed for the marker *glurp*. This is likely due to the overall larger *glurp* PCR fragments (within this study, 537 to 1,111 bp) compared to *msp1* (149 to 275 bp) or *msp2* (205 to 506 bp). *glurp* PCR was also more prone to produce stutter peaks, which cannot easily be distinguished from peaks of minority clones, particularly if a dominant clone is present in a sample. Due to the pronounced stutter peaks in *glurp* PCR, the CE peak height cutoff (applied to prevent mistaking stutter peaks for true fragments) had

TABLE 2 Genotyping outcomes for recurrent parasitemias in 44 paired field samples by individual marker and classification into new infection and recrudescence based on combined results

		Individual marker result[b]			Classification[b]		
Outcome	**n[a]**	***msp2***	***msp1***	***glurp***	**Current approach[c]**	**Proposed approach 1[d]**	**Proposed approach 2[e]**
Clear result							
Recrudescence	22	R	R	R	R	R	R
New infection	8	NI	NI	NI	NI	NI	NI
Intermediate result							
Agreement of *msp1* and *msp2*	7	R	R	NI	NI	R	R
Disparate *msp1* and *msp2* result[e]	4	NI	R	NI	NI	NI	1 NI, 3 R
	2	NI	R	R	NI	R	1 NI, 1 R
	1	R	NI	R	NI	R	NI
Total no. of:							
Recrudescences					22	32	33
New infections					22	12	11

[a]*n*, number of sample pairs per pattern.
[b]NI, new infection; R, recrudescence.
[c]The current approach to define the overall genotyping result as recommended by WHO uses sequential typing—i.e., stopping the genotyping procedure at first indication of a new infection using markers in the order *msp2*, *msp1*, and *glurp*.
[d]New approach 1 proposed in this study is based on forming a consensus result: i.e., the result obtained by 2 out of the 3 markers, *msp1*, *msp2*, and *glurp*.
[e]New approach 2 proposed in this study is based solely on markers *msp1* and *msp2* and compares the compositions of allelic families between pre- and posttreatment samples. A sample pair is only classified as NI if all allelic families differ between between the pre- and posttreatment samples for one of the markers, *msp1* or *msp2*. Detailed genotyping data and classification of these samples are shown in Table S3 in the supplemental material.

to be increased for *glurp* PCR compared to *msp1* or *msp2* PCR. As result, true peaks of minority clones are more likely to fall below the cutoff and thus remain undetected in *glurp* PCR, leading to underestimation of MOI.

An essential observation was the importance of performing simplex allelic family-specific nPCRs for both *msp* markers, whenever increased precision is required. The use of allelic family-specific primers to increase sensitivity and resolution of genotyping in samples containing clones from different allelic families has been proposed previously (2, 7, 18). Our results, however, emphasize that unless nPCRs are performed separately, the gain in minority clone detectability using allelic family-specific primers is marginal. A great gain in sensitivity is observed when nPCRs are performed separately, which justifies the increase in diagnostic cost. Yet, template competition between alleles from the same family cannot be overcome even by simplex nPCR. Marker *glurp* with only one allelic family and longest amplicon sizes can thus be considered the least suitable marker for sensitive discrimination of clones. Studies performed before the adoption of CE-based genotyping methods used PCR-restriction fragment length polymorphism (PCR-RFLP) to distinguish *msp2* clones (19). This protocol did not use family-specific *msp2* primers; therefore, all amplified fragments (ranging from 350 to 750 bp) likely were also subject to amplification bias. Yet, fragments on average were substantially smaller than *glurp* fragments, which ranged from 537 to 1,111 bp in this study.

The significantly lower MOI observed in our field samples using marker *glurp* compared to *msp1* and *msp2* could be due to a lower diversity of *glurp*, but a major factor seems to be suppression of minority clones or long fragments during *glurp* PCR. In light of the severe bias in *glurp* PCR in artificial mixtures of culture strains, new infections identified only by *glurp* genotyping and not by the other two markers are probably attributable to competition in PCR and should be interpreted with care. It is therefore crucial to consider PCR limitations such as amplification bias when designing a strategy for genotyping and data analysis.

The WHO definition of a recrudescent infection is the presence of at least one shared genotype in the compared pre- and posttreatment samples at all loci. A new infection is defined by the presence of only new alleles in the posttreatment sample by at least one of the markers (3). The effects of intra-allelic family competition during PCR demonstrated here suggest a reconsideration of the current recommended genotyping procedure, which stipulates consecutive analysis of the three markers. According to the current strategy, once a new infection is observed by one of the markers the remaining loci are not typed. In contrast, if amplification of both *msp* markers with family-specific simplex nPCRs was mandatory, minority clone detectability would be drastically increased due to (i) the lower likelihood of two clones to harbor alleles of the same *msp1* as well as the same *msp2* family, and (ii) the independence of allele sizes of *msp1* and *msp2* for any given clone, making it unlikely that alleles of both markers will be suppressed during PCR.

Marker *glurp*, despite its shortfalls, might be useful until a more suitable marker is found and validated, as it can provide additional resolution in areas of low transmission with only limited diversity in both *msp* markers. In contrast, in study areas with high mean MOI, *glurp* likely contributes to overestimation of new infections, as later recrudescing minority clones could easily be missed in a multiclonal admission sample. The use of *glurp* as a third marker should hence be critically reviewed, and its application could be restricted to drug trials performed in low-endemicity settings. Furthermore, a more robust definition of "new infection" should be discussed among experts, which could be primarily, or exclusively, based on markers *msp1* and *msp2*. In practice, genotyping of recurrent parasitemias should therefore begin with typing both markers *msp1* and *msp2*. If these two markers agree, *glurp* genotyping would no longer be necessary. In case of a disagreement between *msp1* and *msp2* genotyping outcomes, either *glurp* typing would have to be performed and the "two out of three" rule applied (preferred in low-endemicity settings), or the composition of allelic *msp1* and *msp2* families would be considered (preferred in high-endemicity settings). In the latter approach, final classification as a new infection would require a complete shift of allelic

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families between pre- and posttreatment samples for either *msp1* or *msp2*, as clone competition is abrogated between allelic families if nPCRs are performed as simplex PCRs.

In summary, the following alterations of the current genotyping strategy could be considered: (i) separate nPCRs for each allelic family, (ii) obligate genotyping of the two markers *msp1* and *msp2*, or (iii) classification of recurrent parasitemias based on a consensus result of markers *msp1* and *msp2* first, with disparate *msp1* and *msp2* results resolved using marker *glurp* or, as potentially the best option, by rating *msp1*/*msp2* allelic families for defining a new infection.

In areas where malaria is highly endemic, with frequent multiclone infections, these amendments could affect the outcome of drug efficacy trials in that less recrudescent infections would be misclassified as new infections, and in consequence, overestimation of the efficacy of a tested drug would be reduced.

In the future, such technical limitations might be overcome by the development of single-nucleotide polymorphism-based genotyping techniques, which, in combination with molecular barcoding or next-generation sequencing, might provide highly diverse haplotype markers with sufficient resolution for tasks such as genotyping of recurrent parasitemias (20, 21).

The detectability of a clone in a blood sample is determined by several factors, such as sensitivity of the molecular assay, biological features of the parasite (i.e., sequestration of synchronous clones and fluctuation of densities), and technical limitations. The contribution of each of these factors to a final genotyping result is challenging to disentangle. The improvements suggested for recrudescence typing will not lead to perfect detectability of parasite clones. This method will also not question previous results from earlier applications of genotyping in *in vivo* drug efficacy trials, but it provides a rational basis for a genotyping approach that is less prone to false outcomes by correcting a technical problem.

The WHO/Medicines for Malaria Venture (MMV) consultation (3) had suggested that genotyping should be performed in a sequential manner, starting with marker *msp2* or *glurp*, and then as a third marker, *msp1* was suggested. This decision was based on the workload incurred by markers for which more than one nested PCR needs to be performed. According to the present data, using *glurp* as the first marker in sequential genotyping likely leads to a stronger bias toward a "new infection." However, in many laboratories, *msp2* followed by *msp1* were used as the first and main markers. Therefore, our suggestion of a new genotyping approach will not dramatically change the PCR-corrected outcomes in future clinical trials compared to those of the past, but the precision will be improved. Despite the known shortfalls in genotyping, its application in field trial is indispensable, as in areas of intermediate transmission superinfections during the follow-up period of the trial will yield an incorrect high drug failure rate. In high-transmission settings, the numbers of superinfections are high and clone competition in PCR will be more dramatic. Thus, genotyping is bound to be more accurate than in areas of low and intermediate transmission. However, as multiplicity of infection is declining in most areas of endemicity in the world, the value of genotyping will increase in the future, as samples from the clinical trial areas will have fewer concurrent infections and thus less clone competition.

In conclusion, this study highlights major limitations of *P. falciparum* genotyping using length-polymorphic markers, which are caused by fragment size differences and intra-allelic family competition during nPCR. Our data suggest inclusion of both *msp* markers for genotyping of recurrent parasitemias and raises questions on the use of additional *glurp* genotyping. Relying solely on *glurp* genotyping should be discouraged due to the absence of allelic families and therefore direct template competition between all alleles. Small adjustments to the currently recommended genotyping procedures for clinical trials could significantly improve the accuracy of PCR correction of endpoints in malaria drug or vaccine efficacy trials.

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MATERIALS AND METHODS

Culture strains and field samples. Four *Plasmodium falciparum* culture strains, HB3, 3D7, K1, and FCB1, were selected to represent the allelic families of *msp1* and *msp2* (Table 1). DNA extractions from cultured parasites and blood from a human noninfected volunteer were performed using the QIAamp DNA blood minikit (Qiagen, Germany), and the DNA was stored at 4°C. Parasites were quantified by quantitative PCR targeting the *P. falciparum* 18S rRNA gene using a serial dilution of ring-stage parasites as quantification standards (22, 23). DNA from 2 or 4 strains was mixed in human DNA, to reconstitute human blood, in ratios ranging from 1:1 to 5,000:1. For the minority clone, a minimal concentration of 10 parasite genomes per microliter of blood was chosen to prevent stochastic effects in positivity due to insufficient template in the PCR.

Genotyping was performed on 44 paired anonymous field samples. A sample pair consisted of a pretreatment sample and a sample collected on the day of recurrent parasitemia after antimalarial treatment. Mean time to recurrence was 14 days on average (range, 6 to 28 days). These samples were derived from clinical drug efficacy trials and had been collected at several sites across Africa and Asia. Therefore, a description of "overall" endemicity is not possible for the set of samples used. As a surrogate measure of transmission intensity, the mean multiplicity of infection (MOI) in baseline samples represents a useful parameter in the context of the present article. The mean MOI for marker *msp2* at baseline was 2.3 concurrent infections per PCR-positive individual, with individual MOI ranging from 1 to 7 infections. Ethical clearance for genotyping of these anonymized blood samples was obtained from the Ethikkommission Nordwest-und Zentralschweiz (EKNZ Req-2016-00050).

Genotyping PCR and CE. Primer sequences, PCR mixtures, and cycling conditions are listed in Tables S1 and S2 in the supplemental material. The *msp1* and *msp2* primers correspond to the WHO recommended primers (17), while *glurp* primary PCR (pPCR) and nPCR primers were optimized for CE in the present study. Allelic family-specific simplex and multiplex nPCRs were compared for marker *msp2* using DNA from culture strain mixtures and field samples. Unless stated otherwise, results from allelic family-specific simplex nPCRs are reported. CE of markers *msp1* and *msp2* used the GeneScan 500 LIZ dye size standard (Thermo Fisher Scientific) on an AB3130xl automated sequencer. *glurp* fragment sizing was performed at Macrogen Corp (Amsterdam, The Netherlands) using the GeneScan 1200 LIZ dye size standard (Thermo Fisher Scientific). Fragment analysis was performed with GeneMapper software version 5 (Applied Biosystems). A peak height cutoff for minority clones was set at 10% of the height of the dominant peak for *msp1* and *msp2* genotyping. For genotyping of *glurp*, the cutoff was increased to 20% of the dominant peak to account for the characteristic high stutter peaks.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AAC.01500-16.

TEXT S1, PDF file, 0.8 MB.

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The authors declare they have no conflicts of interest.

I.F. and H.-P.B. conceived the study. C.M. and N.E.H. performed the experiments and analyzed the data. C.M., N.E.H., and I.F. wrote the manuscript. All authors read the manuscript and agreed with the conclusions.

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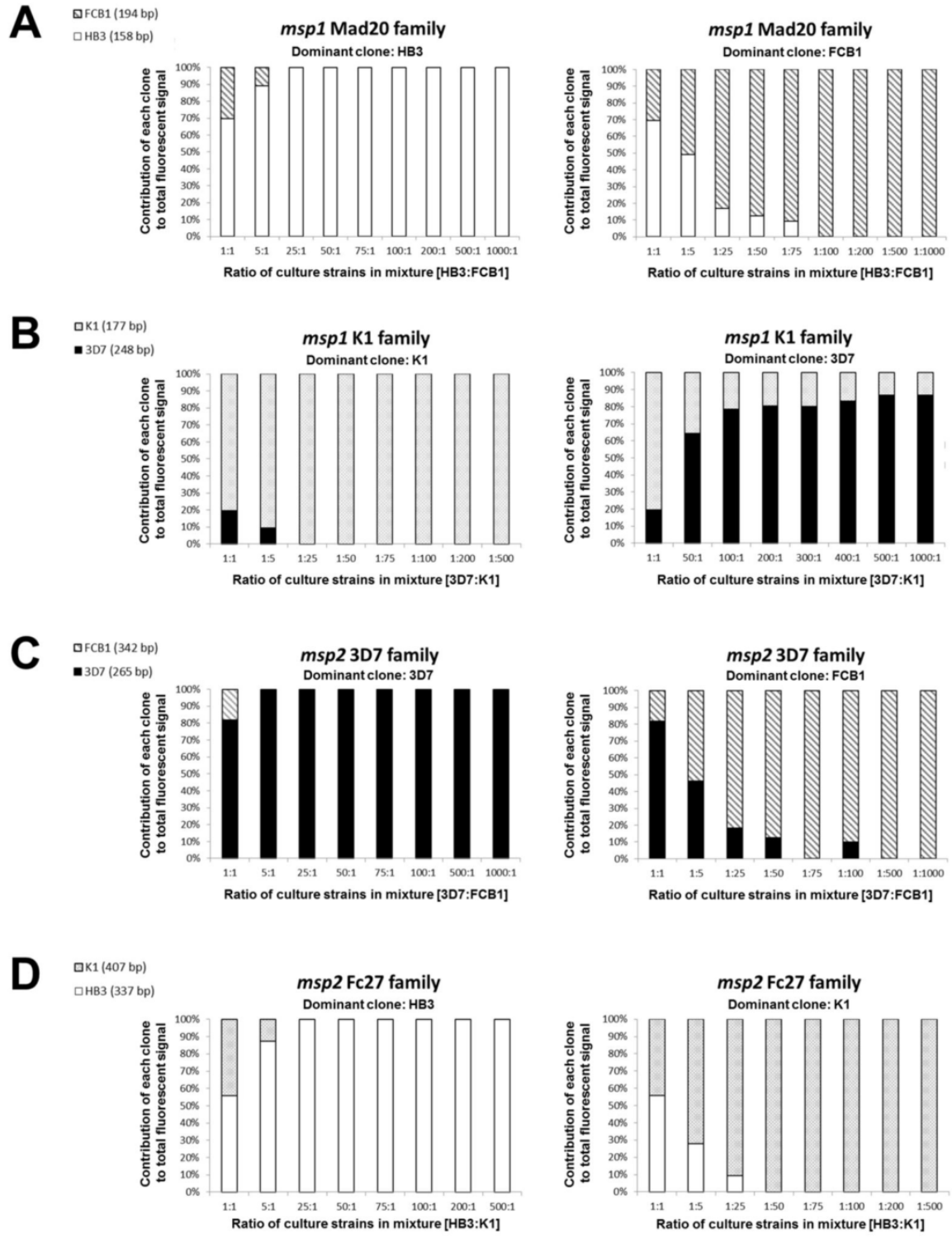
SUPPLEMENT



Supplementary Figure S1: **Proportion of *msp1* and *msp2* fluorescent signals detected during capillary electrophoresis for each clone in two culture strain-mixtures.** Mixed strains carried *msp1* or *msp2* alleles of the same allelic family: (A) Mad20-type *msp1* alleles, (B) K1-type *msp1* alleles, (C) 3D7-type *msp2* alleles, and (D) FC27-type *msp2* alleles. The shorter allele was increasingly dominant in the left panels, the longer allele increasingly dominant in the right panels.

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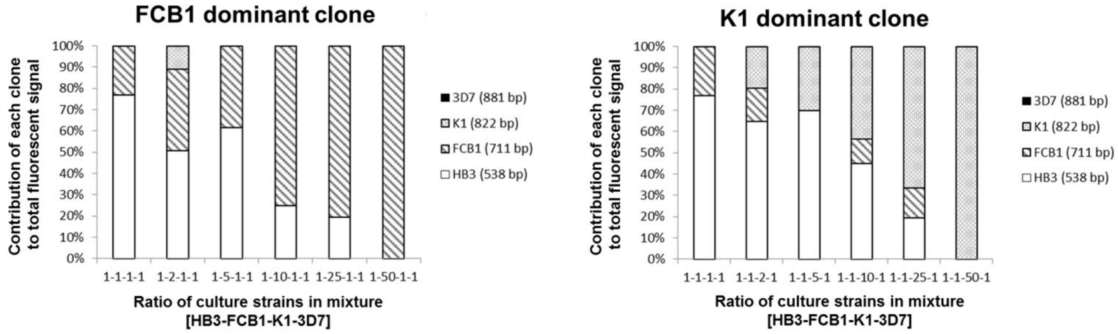

Supplementary Figure S2: Proportions of *glurp* fluorescent signal detected during capillary electrophoresis for each clone in a four culture strain-mixture, with clone FCB1 (2nd-shortest *glurp* allele, left panel) or clone 3D7 (2nd-longest *glurp* allele, right panel) as increasingly dominant clone.

Supplementary Table S1: PCR reaction mixes and thermocyclic profiles

A) PCR genotyping *glurp*

Primary PCR reaction mix and thermo profile	Reagents	Concentration	Volume	Temperature	Time	Cycles
	ddH2O		25.5 µL	94°C	5min	1x
	10x Buffer B	10x	5.0 µL	94°C	1min	|
	dNTPs	2mM	5.0 µL	50°C	1min	25x
	$MgCl_2$	25mM	4.0 µL	72°C	2min	|
	Primer G4	10µM	2.5 µL	72°C	10min	1x
	Primer G5mod	10µM	2.5 µL			
	FIRE Pol®		0.5 µL			
	DNA		5 µL			
			50 µL			

Nested PCR reaction mix and thermo profile	Reagents	Concentration	Volume	Temperature	Time	Cycles
	ddH2O		31.5 µL	94°C	5min	1x
	10x Buffer B	10x	5.0 µL	94°C	1min	|
	dNTPs	2mM	5.0 µL	58°C	1min	30x
	$MgCl_2$	25mM	3.0 µL	72°C	1min	|
	GNF	10µM	2.0 µL	72°C	10min	1x
	G3	10µM	2.0 µL			
	FIRE Pol®		0.5 µL			
	pPCR product		1 µL			
			50 µL			

FIRE Pol® (Solis BioDyne, England)

B) PCR genotyping *msp1/2*

Primary PCR reaction mix and thermo profile (duplex msp1 & msp2)	Reagents	Concentration	Volume	Temperature	Time	Cycles
	ddH2O		28.0 µL	94°C	5min	1x
	10x Buffer B	10x	5.0 µL	94°C	30sec	|
	dNTPs	2mM	5.0 µL	54°C	1min	30x
	$MgCl_2$	25mM	4.0 µL	72°C	1min	|
	Primer Mix M1/M2[a]	10µM	2.5 µL	72°C	5min	1x
	FIRE Pol®		0.5 µL			
	DNA		5 µL			
			50 µL			

[a] Primer mix: M1-OF/M1-OR/M2-OF/M2-OR

msp1 nested PCR reaction mix and thermo profile	Reagents	Concentration	Volume	Temperature	Time	Cycles
	ddH2O		32.5 µL	94°C	5min	1x
	10x Buffer B	10x	5.0 µL	94°C	30sec	|
	dNTPs	2mM	5.0 µL	59°C	1min	30x
	$MgCl_2$	25mM	4.0 µL	72°C	1min	|
	Primer Mix nPCR[b]	10µM	2.0 µL	72°C	5min	1x
	FIRE Pol®		0.5 µL			
	pPCR product		1 µL			
			50 µL			

[b] Primer mix: M1-MF/M1-MR or M1-KF/M1-KR

msp2 nested PCR reaction mix and thermo profile	Reagents	Concentration	Volume	Temperature	Time	Cycles
	ddH2O		33.5 µL	94°C	5min	1x
	10x Buffer B	10x	5.0 µL	94°C	30sec	|
	dNTPs	2mM	5.0 µL	50°C	45sec	30x
	$MgCl_2$	25mM	3.0 µL	72°C	1min	|
	Primer Mix nPCR[c]	10µM	2.0 µL	72°C	10min	1x
	FIRE Pol®		0.5 µL			
	pPCR product		1 µL			
			50 µL			

[c] Primer mix: M5/S-tail or N5/S-tail

Supplementary Table S2: PCR primers

A) glurp genotyping PCR

	Primer ID	T_m	Sequence	CG%	nt	Reference
primary PCR	G4	57.4°C	5'-acatgcaagtgttgatcc-3'	44.4	18	
	G5mod	64.2°C	5'-cagatggtttgggagtaacgtt-3'	45.5	22	
nested PCR	G3	64°C	5'-tgtaggtaccacgggttcttg-3'	52.4	21	
	GNF	65.5°C	(PET) 5'-tgttcacactgaacaattagatttagatca-3'	30	30	Snounou (2002)

B) msp1 genotyping PCR

	Primer ID	T_m	Sequence	CG%	nt	Reference
primary PCR	M1-OF	61.9°C	5'-ctagaagctttagaagatgcagtattg-3'	37	27	Snounou (2002)
	M1-OR	61.1°C	5'-cttaaatagtattctaattcaagtggatca-3'	26.7	30	Snounou (2002)
nested PCR						
Mad20 family	M1-MF	70.1°C	Tail 5'-aaatgaaggaacaagtggaacagctgttac-3'	40	30	Snounou (2002)
	M1-MR	67.6°C	(FAM) 5'-atctgaaggatttgtacgtcttgaattacc-3'	36.7	30	Snounou (2002)
K1 family	M1-KF	65.3°C	Tail 5'-aaatgaagaagaaattactacaaaaggtgc-3'	30	30	Snounou (2002)
	M1-KR	83.2°C	(NED) 5'-gcttgcatcagctggagggcttgcaccaga-3'	60	30	Snounou (2002)

C) msp2 genotyping PCR

	Primer ID	T_m	Sequence	CG%	nt	Reference
primary PCR	M2-OF	58.9°C	5'-atgaaggtaattaaaacattgtctattata-3'	20	30	Snounou (2002)
	M2-OR	62.2°C	5'-ctttgttaccatcggtacattctt-3'	37.5	24	Snounou (2002)
nested PCR						
FC27 family	S-tail-fw	54.3°C	Tail 5'-gcttataatatgagtataaggagaa-3'	28	25	Falk et al. (2006)
	M5-rev	52.2°C	(FAM) 5'-gcattgccagaacttgaa-3'	47.4	19	Falk et al. (2006)
3D7 family	S-tail-fw	54.3°C	Tail 5'-gcttataatatgagtataaggagaa-3'	28	25	Falk et al. (2006)
	N5-rev	60.5°C	(VIC) 5'-ctgaagaggtactggtaga-3'	44.4	18	Falk et al. (2006)

Supplementary Table S3: Genotyping data and classification of 7 samples with discrepant *msp1/msp2* result

Sample pair	*msp1*			*msp2*			*glurp*			Classification	
	D0	DX	Result	D0	DX	Result	D0	DX	Result	Proposed Approach 1	Proposed Approach 2
1	M211	K238 RO33	NI with shift of allelic families	D226 D370	D370 F417	R	766 933	766	R	**R**	**NI**
2	K230 K238 Ro33	Ro33 K212	R	D257 D298 F371	D269	NI	939	819 884	NI	**NI**	**R**
3	Ro33	Ro33	R	F417	D224	NI with shift of allelic families	773	884	NI	**NI**	**NI**
4	M219 M202 K177 K203 K212 K230 K284 Ro33	M219 K212 Ro33	R	D226 D234 D271 D282 D291 F408	D241 D250 D375 F337 F382	NI	602	832	NI	**NI**	**R**
5	K203 K212 Ro33	Ro33 K257	R	D286 D352	F337	NI with shift of allelic families	939	939 1111	R	**R**	**NI**
6	M193 M202 K239 Ro33	Ro33 K194 K212	R	D253 F371 F454	D343 F348 F419	NI	822 939	831 1052 1111	NI	**NI**	**R**
7	M193	M193	R	D300 F371	F337	NI	602 830	602	R	**R**	**R**

Chapter 7:

General Discussion

General Discussion

The central theme of this thesis was the molecular epidemiology and infection dynamics of submicroscopic and asymptomatic *P. falciparum* infections, whose relevance for malaria elimination has received much attention recently (Bousema et al., 2014; Okell et al., 2012). This chapter focuses on the translational research aspects of this thesis and their significance of for planning of intervention and surveillance strategies for malaria elimination. It further discusses limitations of this thesis and gives directions for future research.

7.1 Relevance of submicroscopic and asymptomatic infections for malaria control

Accurate estimates of malaria transmission are imperative to inform malaria control programs; however, the surveillance strategy used and choice of diagnostics have a major impact on the accuracy of the obtained estimate. Monitoring of clinical malaria cases at health facilities is easier implemented than active screening of large populations, but it ignores asymptomatic infections that play a major role in sustaining transmission (Bousema et al., 2012a; Bousema et al., 2014; Cheng et al., 2015; Churcher et al., 2013; Okell et al., 2009, 2012). In our study population, *P. falciparum* was the most frequent species detected in clinical cases although asymptomatic infections with *P. vivax* were much more common than with *P. falciparum* (chapter 3). Monitoring of clinical cases only, even if based on active case detection like in the Albinama cohort, thus has severe shortcomings to reflect species composition of the asymptomatic reservoir in settings where several species co-exist. This is because immunity is developed at different rate to different species (chapter 3) (Karyana et al., 2008; Koepfli et al., 2013; Lin et al., 2010; Maitland et al., 1996; Michon et al., 2007; Phimpraphi et al., 2008).

Little evidence exists on the proportion of infections that remain purely asymptomatic until naturally cleared by the immune system and that would thus completely evade clinical surveillance. Longitudinal tracking of *P. falciparum* and *P. vivax* clones in the Albinama cohort allowed estimating the infection rate and the proportion of purely asymptomatic infections. Within eight months after the initial treatment, 40% and 20% of children were re-infected with *P. vivax* and *P. falciparum*, respectively, without ever presenting any symptoms, as opposed to 20% of children who became symptomatic (chapter 3). The majority of clones detected in samples from symptomatic malaria episodes were not detected previously in the same child, suggesting a recent new infection as cause for the episode (Figure 1). Assuming that each episode was caused by a single infecting clone, only 4% of 849 *P. vivax* and 20% of 342 *P. falciparum* genetically distinct new infections proceeded to symptomatic disease. Asymptomatic infections hence make up the vast majority particularly of *P.*

vivax malaria in semi-immune school-aged PNG children, with even larger proportions to be expected in adults (Kasehagen et al., 2006; Koepfli et al., 2015).

Figure 1. Proportion of *P. falciparum* and *P. vivax* asymptomatic clones versus clones detected in episodes in the Albinama study. Asymptomatic clones are defined as detected only in asymptomatic samples but not in an episode sample of a child. The majority of clones observed in episode samples were new infections not observed in the preceding samples from the same child ("new infections"). Some clones observed in episodes had been detected before in the same child ("persisting clones"). The proportion of asymptomatic clones versus clones in episodes is visualized by age group.

Although long debated, it is now clear that the long-term health and societal consequences of chronic, asymptomatic malaria infections outweigh their potential benefits for the human host (Chen et al., 2016; Greenwood, 1987) such as conferring anti-malarial immunity and mitigating the severity of super-infections (Smith et al., 1999). Indeed, we found indications that higher exposure to *P. falciparum* conferred some protection against symptomatic *P. falciparum* malaria in the Albinama cohort; however, this did not outweigh the risks associated with frequent infection. Children living in the highest endemic villages still carried the main burden of disease (chapter 3). Our results therefore support the need for treatment of asymptomatic infections, which is increasingly appreciated as a cornerstone of successful malaria elimination strategies (Bousema et al., 2014; malERA Consultative Group on Diagnoses and Diagnostics, 2011).

Mass drug administration (MDA) includes treatment of all asymptomatic carriers and, if high coverage can be achieved, may serve as a valuable elimination tool despite fears of accelerating the development of drug resistance (Newby et al., 2015). A variety of test-and-treat strategies are being evaluated to mitigate the risks of MDA (Sturrock et al., 2013), however, are intrinsically limited by the sensitivity of the diagnostic test used to detect low-density malaria infections (Cook et al., 2015a; Mosha et al., 2013; Tiono et al., 2013). RDTs are often used in test-and-treat interventions and have a limit of detection comparable to LM, which fails to detect half of *P. falciparum* infections and two thirds of *P. vivax* infections world-wide (Bousema et al., 2014; Cheng et al., 2015; Okell et al., 2009,

2012). Also in our cross-sectional study in high-endemic Rufiji/Tanzania, as well as in the medium-endemic Albinama cohort in PNG, more than half of infections with *P. falciparum* (and *P. vivax*) were not detected by LM (chapters 2 and 3). In Rufiji, application of two ultra-sensitive qPCR assays uncovered a much larger extent of submicroscopic infections than previously anticipated, as almost a third (48/169) of submicroscopic infections were only identified by ultra-sensitive PCR but not standard PCR (chapter 2). Similar observations of ultra-low density infections were made for *P. falciparum* and *P. vivax* infections along the Thai/Myanmar border, Cambodia and Vietnam using high-volume blood sampling-ultra sensitive PCR (Imwong et al., 2014, 2015a, 2015b). The extent of asymptomatic malaria is therefore underestimated even by standard PCR, and consequentially even more so by RDT or LM. This casts further doubt on the usefulness of test-and-treat approaches for malaria elimination. Whether even more malaria infections would be found if sensitivity of detection increased further, and which level of sensitivity is required for sensible diagnosis of parasitemia in test-and-treat interventions remains to be investigated.

The relevance of detecting and treating ultra-low density infections is questionable since transmission to mosquitoes occurs less frequently at low density of blood-stage parasites (Ross et al., 2006) or gametocytes (Churcher et al., 2013) compared to high-density infections. However, until more is known on the fate of ultra-low density infections in the human host, aiming for the most sensitive detection method available seems prudent if accurate estimates of parasite prevalence are required.

In their current setup, the two ultra-sensitive assays for detection of *P. falciparum* are mainly applicable for monitoring parasite prevalence in random community samples or cross sectional surveys in a central core facility with the required equipment. Because PCR requires DNA extraction and temperature cycling it is currently not applicable for point-of-care (POC) diagnosis. However, the rapid development of microfluidic devices (Tay et al., 2016) may soon enable field-based PCR using lab-on-a-chip or battery-operated devices (Nair et al., 2016) if the need for DNA extraction can be overcome. Alternatively, isothermal amplification methods such as LAMP seem easier field-applicable (Cook et al., 2015b; Oriero et al., 2015), although concerns exist on the specificity and false-positivity rate of LAMP (Felger/Antunes-Moniz, personal communication). Other strategies aim at improving performance of RDT by concentration of the sample material (Pereira et al., 2015). In light of this variety of approaches, achieving sensitive POC malaria diagnosis seems feasible within the near future. These may have major implications on the design of malaria elimination strategies particularly in areas where emerging drug resistance renders MDA the undesired strategy towards screen-and-treat campaigns.

7.2 Evaluating heterogeneity in transmission for targeting of interventions

Heterogeneity in malaria transmission is observed on global (Gething et al., 2011, 2012), regional, and local scale down to the household level in areas of unstable transmission as well as in high-endemic areas (Bejon et al., 2014; Gaudart et al., 2006; Hu et al., 2016; Rosas-Aguirre et al., 2015; Sissoko et al., 2015). Different factors such as presence of water bodies and mosquito breeding sites, vegetation, climate, altitude, human behavioral factors, housing structure and genetic factors influence local malaria transmission and exposure of the individual to mosquito bites (Bousema et al., 2012b). This heterogeneity is recognized as a major challenge to malaria control, but vice versa also appreciated as opportunity to maximize intervention effectiveness. This can be achieved by specific targeting of interventions to pockets of highest transmission that may fuel malaria transmission in a wider area (Bousema et al., 2012b). While theoretically promising, the evidence base comparing spatially targeted interventions to untargeted community-based approaches is thin. A recent cluster-randomized controlled trial in Kenya evaluated the effects of focally targeted larviciding, LLIN distribution, IRS, and focal mass drug administration against the standard national malaria control policy and found no sustained effect on *P. falciparum* prevalence outside the spatial limits of the targeted areas (Bousema et al., 2016). Also mathematical modeling suggests that effects of vector control measures are higher when interventions are spatially distributed over large areas (Lutambi et al., 2014), and that drug campaigns are less effective when selectively targeted to high-transmission areas compared to untargeted approaches (Gerardin et al., 2016).

The Albinama cohort study took place in the East Sepik/PNG approximately 2 years after a nationwide distribution of LLINs (Hetzel et al., 2012). *P. falciparum* and *P. vivax* transmission varied drastically between villages in spite of the fact that the participating children spent on average more than 90% of nights protected by a LLIN in all villages (chapter 3). Similar micro-geographical heterogeneity in malaria transmission has been described in several other studies conducted before the countrywide distribution of LLINs in various areas in PNG (Cattani et al., 1986; Genton et al., 1995; Mueller et al., 2009; Müller et al., 2003). A main challenge is explaining why this heterogeneity in malaria transmission can persist despite the high coverage with LLINs, and requires identifying the underlying patterns of human behavioral factors and vector characteristics such as species composition, biting behavior or vectorial capacity. This data is crucial to inform future planning of control strategies in PNG, which will need to address the micro-geographical differences in malaria transmission in absence of high-resolution data for most areas of PNG. Based on the geographical patterns observed in children in the Albinama cohort, where high episode incidence marked villages with highest transmission, reactive case detection (RCD) may be a promising approach to target geographically heterogeneous malaria in PNG. In RCD, clinical malaria cases presenting to a health facility trigger active screening of individuals living in a pre-defined radius around the origin of the index case and treatment of all parasite carriers. RCD has shown promising results in Zambia and

Swaziland (Larsen et al., 2015; Sturrock et al., 2013), but in contrast, effectiveness of RCD was limited in settings where exposure does not occur within the community (Hustedt et al., 2016; Littrell et al., 2013).

Parasite prevalence is the most widely accepted metric to measure malaria transmission in cross-sectional studies (Gething et al., 2011, 2012; Tusting et al., 2014), which are the most commonly performed type of study within malaria control programs. Other parameters such as the multiplicity of infection (MOI) or the proportion of single clone infections are based on parasite genotyping, and realistically reflect differences in malaria transmission between countries or after anti-malarial interventions (Anderson et al., 2000; Ibara-Okabande et al., 2012; Nkhoma et al., 2013). The usefulness of these three cross-sectional parameters for assessing small-scale variations in malaria transmission was validated in the Albinama cohort using *Pf*-$_{mol}$FOI and *Pv*-$_{mol}$FOB (after PQ treatment) as a longitudinal, molecular gold standard of transmission from mosquitoes. By village, *P. falciparum* parasite prevalence by PCR correlated almost perfectly with *Pf*-$_{mol}$FOI (Figure 2). Also for *P. vivax*, prevalence showed the best correlation with *Pv*-$_{mol}$FOB by village (after PQ treatment), but generally correlations were weaker than for *P. falciparum* due to the contribution of relapses that dampened variations in cross-sectional parameters between villages (Figure 2). Parasite prevalence by PCR, the easiest obtainable molecular metric in cross-sectional studies, therefore closely reflects small-scale variations in transmission and can be used for identifying pockets of high transmission and investigating the underlying factors of transmission heterogeneity.

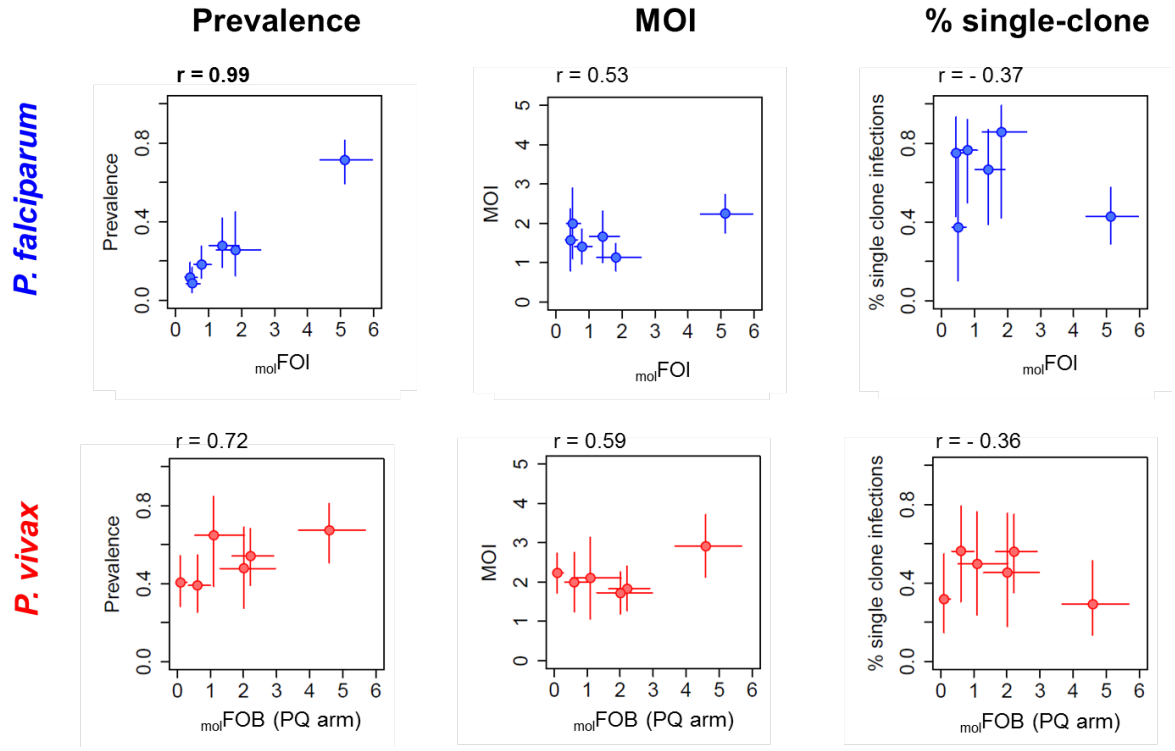

Figure 2. Correlation of surrogate markers of transmission by village. Prevalence, MOI and the proportion of single-clone infections can be assessed in cross-sectional studies. Measuring $_{mol}$FOI/$_{mol}$FOB requires longitudinal studies but provides more accurate estimates of transmission.

7.3 PQ treatment in malaria control: risks and benefits

PQ treatment is currently advocated for two applications in malaria control: (i) for clearance of *P. vivax* and *P. ovale* hypnozoites and prevention of relapse, for which a 14-day administration schedule á 0.25 mg/kg is required (WHO, 2015); and (ii) for reduction of post-treatment *P. falciparum* gametocyte carriage using a single dose of PQ along with ACT (White, 2013; World Health Organization, 2015). Clinical use of PQ is severely limited due to its hemolytic effect in individuals with reduced activity of the red blood cell glucose-6-phosphatase dehydrogenase (G6PD) (Alving et al., 1956), which affects up to 30% of the population in malaria-endemic areas in Africa (Howes et al., 2012). In PNG, the prevalence of G6PD-deficiency varies throughout the country and ranges from 1% along the south coast to 15% in the East Sepik (Howes et al., 2012).

Treatment of confirmed *P. vivax* and *P. ovale* malaria in G6PD-normal patients with ACT plus PQ is now recommended at all endemicity levels by WHO, acknowledging a previous underestimation of malaria morbidity attributable to relapses (WHO, 2015). Results from the Albinama cohort support the validity of this revised recommendation. Our results even suggest mass drug administration of PQ in areas such as PNG, where the fast-relapsing tropical strain of *P. vivax* is endemic and relapses account for four out of five *P. vivax* infections; and where the majority of the population carries dormant hypnozoites (Robinson et al., 2015). The detailed investigation of patterns in malaria infection and disease the Albinama cohort (chapter 3) showed that frequent relapses remain the main source of *P. vivax* morbidity in 5-10 year old, semi-immune children in PNG. At this age, children in the East Sepik have acquired substantial clinical immunity to *P. vivax* over years of exposure with at least 5 (chapter 3, in 5-10 year old children) to 14 (Koepfli et al., 2013; in 1-4 year old children) genetically distinct blood-stage infections per year. Due to the genetic diversity of these past *P. vivax* infections, it is conceivable that semi-immune children in PNG may be equally able to control new *P. vivax* infections from infective bites and relapsing infections. This is because relapses are not always genetically identical to the primary infection but can also be meiotic siblings (Bright et al., 2014).

Our results therefore emphasize the necessity of PQ treatment for reducing *P. vivax* infection and morbidity in PNG. Currently, the absence of a reliable and affordable RDT for G6PD deficiency testing as well as the poor quality of PQ available in the public as well private sector in PNG (Hetzel et al., 2014) are the main obstacles preventing effective treatment of the *P. vivax* hynozoite reservoir. However, new RDTs for point-of-care diagnosis of G6PD deficiency are currently being tested in field settings (Adu-Gyasi et al., 2015; Kim et al., 2011; Ley et al., 2015) that could be used to mitigate the risk of hemolysis caused by PQ treatment in health facilities or during MDA campaigns. Tafenoquine, an 8-aminoquilone with better efficacy than PQ, can be given as single dose and is currently being tested in phase 3 trials (Rajapakse et al., 2015). In contrast to the 14-day PQ treatment regimen required for effective clearance of *P. vivax* hynozoites, the arrival of Tafenoquine would therefore drastically simplify the implementation of MDA using hypnozoite-clearing drugs.

Reducing the prevalence of *P. vivax* in areas where it is co-endemic with other *Plasmodium* species may have secondary effects particularly on *P. falciparum* infections due to altered within-host cross-species interactions. *P. falciparum* and *P. vivax* seem to mutually reduce prevalence of the other species and *P. vivax* infections seem to lower the risk of *P. falciparum* clinical disease (Mayxay et al., 2004). Also in the Albinama cohort we observed that a concurrent *P. vivax* infection was associated with reduced *P. falciparum* parasite densities (chapter 3), a reduced risk for *P. falciparum* episodes (chapter 3) and reduced *P. falciparum* gametocyte carriage (chapter 5; similar to Koepfli et al., 2015). It can therefore be hypothesized that by reducing *P. vivax* infections in the population using intensified PQ treatment, *P. falciparum*-associated malaria episodes and *P. falciparum* gametocyte carriage may temporarily and transiently increase. However, with the drastic decline in prevalence of both species in PNG, mixed *P. falciparum*/*P. vivax* infections have already become highly infrequent (Hetzel et al., 2015; Koepfli et al., 2015) and their relevance for control is thus likely to wane even more in the future.

In contrast to its importance for *P. vivax* control, the use of PQ as gametocidal drug in treatment of *P. falciparum* malaria has to be critically reviewed. Currently, PQ is the only licensed drug for clearance of mature *P. falciparum* gametocytes, which can circulate in the blood for three to six weeks after clearance of asexual stages by commonly used anti-malarial drugs and thus may be transmitted to mosquitoes (Bousema and Drakeley, 2011; Karl et al., 2015). In Ugandan children with uncomplicated malaria, a single dose of PQ (at 0.4 or 0.75 mg/kg) co-administered with artemether-lumefantrine (AL) shortened mean post-treatment gametocyte carriage from 12 to 6 days compared to AL alone (Eziefula et al., 2014). However, mathematical modeling suggests that due to the short half-life of PQ, administrating PQ together with blood-stage treatment does not effectively reduce infectivity (Lawpoolsri et al., 2009). This is because no residual PQ will be present at days 7-10 after the clinical episode, when gametocytes that were already sequestered in the bone marrow at the time of blood-stage treatment will be released into the blood stream (Lawpoolsri et al., 2009).

The long maturation time of *P. falciparum* gametocytes is the reason why gametocyte carriage in symptomatic infections is often attributed not to the illness-causing infection, but rather a pre-existing chronic infection (Karl et al., 2016; Nacher et al., 2002; Price et al., 1999; von Seidlein et al., 2001; Sowunmi et al., 2004). Also in the Albinama cohort, fever was not associated with higher *P. falciparum* gametocyte positivity or density, and 79% of *P. falciparum* gametocyte detections occurred in asymptomatic infections (chapter 5). Our results therefore emphasize that single-dose PQ treatment of *P. falciparum* episodes alone is unlikely to accomplish interruption of transmission of *P. falciparum*, unless the asymptomatic reservoir is targeted simultaneously. Single-dose PQ treatment of *P. falciparum* episodes can only achieve a population-wide reduction in *P. falciparum* transmission if coverage is high and in areas where malaria infectious are predominantly symptomatic (World Health Organization, 2015). The impact of single-dose PQ for interruption of transmission is therefore

questionable particularly in low-transmission settings, where a large proportion of infections are asymptomatic (Okell et al., 2009). A recent trial in asymptomatic Ugandan children showed that gametocyte carriage was significantly shorter after treatment with AL plus PQ compared to AL alone; however, in that trial infectivity to mosquitoes was very low even after AL-only treatment (Gonçalves et al., 2016). This suggests that ACT treatment of asymptomatic, often low-density infections may in itself be sufficient to reduce gametocyte densities to sub-transmissive levels, and that the benefit of adding PQ to ACT treatment of asymptomatic *P. falciparum* malaria may be marginal. Similar conclusions were reached using mathematical modeling of MDA campaigns, which described that adding PQ to MDA with AL or dihydroartemisinin-piperaquine only conferred a small additional reduction in parasite prevalence in the population compared to MDA without PQ (Gerardin et al., 2015).

7.4 Limitations of this thesis and outlook

7.4.1 Effects of length-dependent fragment competition during genotyping PCR

One of the main objectives of this thesis was the determination of *P. falciparum* and *P. vivax* $_{mol}$FOI/$_{mol}$FOB in the Albinama cohort. Estimates of $_{mol}$FOI/$_{mol}$FOB were calculated by longitudinally tracking parasite clones defined by genotyping of length-polymorphic markers. However, in a later validation we found that length-polymorphic markers are subject to severe amplification bias during PCR with shorter fragments outperforming larger fragments (chapter 6). Competition between templates of different length was strongest between alleles of the same family and when nPCR was multiplexed (chapter 6). Genotyping of *P. falciparum* in the Albinama cohort used marker *Pf-msp2* with multiplex nPCR, which in light of our validation was suboptimal for detection of minority clones. Marker *Pv-msp1F3* for genotyping of *P. vivax* infections in the Albinama cohort possesses only one allelic family, leading to direct competition of all alleles. Allele sizes of *Pv-msp1* (147-445bp) were comparable to *Pf-msp2* (206-619bp), and hence the amplification bias during *Pv-msp1F3* nPCR is likely similar or even more severe compared to *Pf-msp2* nPCR, for which two allelic families exist. Amplification of *Pv-msp1F3* in a multiplex pPCR with alternative marker *Pv-MS16* may have further aggravated bias during PCR. However, our validation showed that template competition was almost completely abrogated between *Pf-msp2* alleles of different families when nPCRs were performed as simplex PCRs (chapter 6). This indicates that all alleles were amplified during pPCR and that template competition occurs mainly during extensive re-amplification in nPCR. Also an earlier analysis of *Pf-msp2* and *Pv-msp1F3* clone detectability using the same PCR protocols found similar minority clone detectability for both markers (*Pf-msp2*, 79%; *Pv-msp1F3*, 73%; Koepfli et al., 2011). *P. vivax* infections were generally characterized by lower parasite density and higher number of concurrent clones compared to *P. falciparum* infections in the Albinama cohort (chapter 3), which may have led to lower *P. vivax* minority clone detectability relative to *P. falciparum*.

However, the fact that $_{mol}$FOI/$_{mol}$FOB are obtained from a longitudinal sequence of samples may reduce the impact of bias during PCR on estimates of $_{mol}$FOI/$_{mol}$FOB. Clone composition in chronic infections likely varies between subsequent samples, with some infections being cleared and others newly establishing themselves in the blood stream. Furthermore, fluctuations in clonal density during the course of an infection may lead to detection of different majority clones in consecutive samples, even if overall the same clones are present. When several consecutive samples are concerned, failure to detect an infecting clone may be attributable more to the frequency of sampling versus infection duration (chapter 4). In addition, the discriminatory power of the genotyping marker due to dominant alleles (>20% of allele detections as observed for *Pf-msp2* and *Pv-msp1F3* in the Albinama cohort) may have a more substantial effect on $_{mol}$FOI/$_{mol}$FOB estimates than bias during PCR, as re-infections with the same genotype will go undetected.

Nonetheless, in light of the pitfalls of length polymorphic markers, research should be intensified on alternative genotyping methods based on single-nucleotide-polymorphisms such as barcoding (Galinsky et al., 2015), high-resolution melt curve analysis (Daniels et al., 2015) or next-generation sequencing (Campino et al., 2011). Next-generation sequencing-based methods are particularly promising because they allow high-throughput parallel genotyping of several markers in hundreds of samples. In large surveillance studies, drug resistance markers could possibly be included along with genotyping markers without an increase in cost. However, the relative limit of detection of minority clones using deep sequencing remains to be validated, especially when amplification reactions are multiplexed.

7.4.2 Detection of ultra-low density *P. vivax* infections

Due to the characteristic lower density of *P. vivax* compared to *P. falciparum* infections, ultra-sensitive assays for reliable detection of low-density *P. vivax* infections would be of great use for both surveillance as well as monitoring of intervention efficiency. Identification of multi-copy sequences in the *P. vivax* genome suitable for qPCR design, similar to the TARE-2 and *var*ATS assays developed for *P. falciparum* (chapter 2), was not successful within the framework of this thesis. No telomeric repeat structures have been described for *P. vivax* (Carlton et al., 2008), and no repeats were found by searching the *P. vivax* telomeres of strain Sal1 (http://plasmodb.org) and the recently available P01 isolate (http://www.genedb.org/ Homepage/PvivaxP01) using repeat finder software. *Vir*/*pir* genes, ortholog to *P. falciparum var* genes, are a multi-gene family with 346 members that are grouped into 12 clusters based on amino acid sequence homology (Lopez et al., 2013; Merino et al., 2006; del Portillo et al., 2001). Although the presence of a conserved transmembrane domain has been described (Carlton et al., 2008), DNA sequences of *vir/pir* genes exhibited only little conservation between clusters, thereby impeding the design of primers that amplify several clusters. Also within clusters, *vir/pir* DNA sequences were too variable even for degenerate primer design. Other multi-gene families

exist in *P. vivax,* but comprise less than 56 members (Tachibana et al., 2012) with low DNA sequence conservation.

Similar to *P. falciparum*, targeting *P. vivax* mitochondrial DNA may increase PCR sensitivity (Putaporntip et al., 2011). Indeed, this strategy seemed promising in initial experiments, but was not pursued further due to time constraints. Finally, RNA-based detection of highly abundant 18S rRNA transcripts in *P. vivax* infections may be a valuable alternative to DNA based detection. Due to the lower parasite densities and thus lower abundancy of *P. vivax* 18S rRNA transcripts compared to *P. falciparum*, this strategy may be less prone to cross-contamination between samples, which is one of the major drawbacks of *P. falciparum* RNA-based detection (Wampfler et al., 2013). Nonetheless, RNA-based detection requires more elaborate sampling strategy, extraction method, sample handling and storage compared to DNA-based detection. The design of a robust, ultra-sensitive assay for detection of *P. vivax*, preferentially DNA-based, therefore remains a major challenge for future research.

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Appendix

Contributions to the following publications were made during the course of this thesis:

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Chugh, M., Scheurer, C., Sax, S., Bilsland, E., van Schalkwyk, D.A., Wicht, K.J., Hofmann, N., Sharma, A., Bashyam, S., Singh, S., et al. (2015). Identification and deconvolution of cross-resistance signals from anti-malarial compounds using multidrug-resistant Plasmodium falciparum strains. Antimicrob. Agents Chemother. *59*, 1110–1118.

PLOS | MEDICINE

RESEARCH ARTICLE

Strategies for Understanding and Reducing the *Plasmodium vivax* and *Plasmodium ovale* Hypnozoite Reservoir in Papua New Guinean Children: A Randomised Placebo-Controlled Trial and Mathematical Model

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Abstract

Background

The undetectable hypnozoite reservoir for relapsing *Plasmodium vivax* and *P. ovale* malarias presents a major challenge for malaria control and elimination in endemic countries. This study aims to directly determine the contribution of relapses to the burden of *P. vivax* and *P. ovale* infection, illness, and transmission in Papua New Guinean children.

Methods and Findings

From 17 August 2009 to 20 May 2010, 524 children aged 5–10 y from East Sepik Province in Papua New Guinea (PNG) participated in a randomised double-blind placebo-controlled trial of blood- plus liver-stage drugs (chloroquine [CQ], 3 d; artemether-lumefantrine [AL], 3 d; and primaquine [PQ], 20 d, 10 mg/kg total dose) (261 children) or blood-stage drugs only (CQ, 3 d; AL, 3 d; and placebo [PL], 20 d) (263 children). Participants, study staff, and investigators were blinded to the treatment allocation. Twenty children were excluded during the treatment phase (PQ arm: 14, PL arm: 6), and 504 were followed actively for 9 mo. During the follow-up time, 18 children (PQ arm: 7, PL arm: 11) were lost to follow-up. Main primary and secondary outcome measures were time to first *P. vivax* infection (by qPCR), time to

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Abbreviations: AL, artemether-lumefantrine; CQ, chloroquine; DHA-PIP, dihydroartemisinin-piperaquine; DOT, directly observed treatment; G6PD, glucose-6-phosphate dehydrogenase; HR, hazard ratio; IRR, incidence rate ratio; LM, light microscopy; MDA, mass drug administration; $_{mol}$FOB, molecular force of blood-stage infection; MSAT, mass screening and treatment; NB reg, negative binomial regression; PH, proportional hazards; PL, placebo; PNG, Papua New Guinea; PQ, primaquine; qPCR, quantitative real-time PCR; qRT-PCR, quantitative reverse transcription PCR; RDT, rapid diagnostic test.

first clinical episode, force of infection, gametocyte positivity, and time to first *P. ovale* infection (by PCR). A basic stochastic transmission model was developed to estimate the potential effect of mass drug administration (MDA) for the prevention of recurrent *P. vivax* infections. Targeting hypnozoites through PQ treatment reduced the risk of having at least one qPCR-detectable *P. vivax* or *P. ovale* infection during 8 mo of follow-up (*P. vivax*: PQ arm 0.63/y versus PL arm 2.62/y, HR = 0.18 [95% CI 0.14, 0.25], $p < 0.001$; *P. ovale*: 0.06 versus 0.14, HR = 0.31 [95% CI 0.13, 0.77], $p = 0.011$) and the risk of having at least one clinical *P. vivax* episode (HR = 0.25 [95% CI 0.11, 0.61], $p = 0.002$). PQ also reduced the molecular force of *P. vivax* blood-stage infection in the first 3 mo of follow-up (PQ arm 1.90/y versus PL arm 7.75/y, incidence rate ratio [IRR] = 0.21 [95% CI 0.15, 0.28], $p < 0.001$). Children who received PQ were less likely to carry *P. vivax* gametocytes (IRR = 0.27 [95% CI 0.19, 0.38], $p < 0.001$). PQ had a comparable effect irrespective of the presence of *P. vivax* blood-stage infection at the time of treatment ($p = 0.14$). Modelling revealed that mass screening and treatment with highly sensitive quantitative real-time PCR, or MDA with blood-stage treatment alone, would have only a transient effect on *P. vivax* transmission levels, while MDA that includes liver-stage treatment is predicted to be a highly effective strategy for *P. vivax* elimination. The inclusion of a directly observed 20-d treatment regime maximises the efficiency of hypnozoite clearance but limits the generalisability of results to real-world MDA programmes.

Conclusions

These results suggest that relapses cause approximately four of every five *P. vivax* infections and at least three of every five *P. ovale* infections in PNG children and are important in sustaining transmission. MDA campaigns combining blood- and liver-stage treatment are predicted to be a highly efficacious intervention for reducing *P. vivax* and *P. ovale* transmission.

Trial registration

ClinicalTrials.gov NCT02143934

Introduction

Renewed intensification of global malaria control efforts over the last 15 years have been successful in significantly reducing the global burden of malaria, with many countries in the Asia-Pacific and the Americas seeing a reduction of >90% in the number of clinical cases [1]. As a consequence, 34 countries are actively attempting to eliminate malaria, and many others are considering doing so in the near future [2]. In 2013, political leaders in Central American and East Asian countries, representing >60% of the global population, declared their intention to eliminate malaria in their regions by 2020 and 2030, respectively [3,4]. In parallel to the reduction in overall incidence, a pronounced shift in species composition has been observed, with *Plasmodium vivax* now the predominant *Plasmodium* species in the vast majority of countries outside Africa [2], accounting for 90%–100% of clinical cases in countries such as Guatemala, Brazil, Solomon Islands, and Vanuatu [1].

Despite a significant reduction in *P. vivax* malaria in the last 20 years, *P. vivax* has several biological characteristics that enable it to evade existing control and elimination efforts, which are mainly directed against *P. falciparum* blood stages [5,6]. First and foremost is the ability of *P. vivax* to relapse weeks, months, and years after a primary infection, via a poorly understood reactivation of dormant hypnozoite stages in the liver [7]. These stages cannot be detected with currently available diagnostic tools and are not cleared upon treatment with routinely administered anti-malarial drugs, unless primaquine (PQ)—a drug that requires at least 7–14 d of administration and can cause severe haemolysis in people with glucose-6-phosphate dehydrogenase (G6PD) deficiency—is added to the treatment [8].

In *P. vivax*–endemic regions, hypnozoites constitute a reservoir of diverse *P. vivax* strains that will cause blood-stage infections at a later point in time [9,10]. They are therefore likely not only to account for a high number of *P. vivax* blood-stage infections but also to contribute a high number of concurrently circulating parasite clones in the blood. Despite recent advances in the molecular detection and genotyping of *P. vivax* parasites [11], it is not yet possible to determine whether an infection detected in the blood of an individual originated from a new sporozoite inoculation or is a relapse from a hypnozoite. *P. vivax* produces gametocytes rapidly and continuously over the course of an infection [10], and even low-density infections are thus potentially infectious. If all clones, relapse-derived and newly acquired, produce gametocytes concurrently, these infections can potentially be transmitted together, and the chance for sexual recombination in the mosquito is greatly increased, thus contributing to the maintenance of high *P. vivax* genetic diversity even at low transmission levels [12–14].

P. vivax is thus considered one of the major challenges for elimination of malaria outside Africa [15]. Better data and tools are urgently required to estimate the *P. vivax* hypnozoite reservoir, quantify the relapse burden, better understand potential relapse triggers, and develop the most appropriate public health intervention strategies [15–17].

In malaria-endemic areas of Papua New Guinea (PNG), where four of the five human *Plasmodium* species coexist, *P. vivax* predominates as the cause of infection and illness in young children [18,19] and is gradually replaced by *P. falciparum* as the main cause of disease in older children and adults [20], although *P. vivax* infections remain common throughout childhood and into adulthood, with a prevalence of 13%–36% in cross-sectional surveys conducted in PNG between 2005 and 2010 [21–24]. *P. ovale* and *P. malariae* are much less common, with a 2010 survey revealing a prevalence of 0.1% and 1.3%, respectively (by quantitative real-time PCR [qPCR]), and are mostly observed in mixed-species infections [22,25]. The PNG standard anti-malarial treatment is artemether-lumefantrine (AL), which acts against the blood stage of the parasite but does not eliminate hypnozoites. In the absence of a nationwide, cost-effective strategy of screening for G6PD, PQ treatment for clearing liver stages, although recommended for G6PD-normal patients, is rarely given. Consequently, relapses are expected to contribute significantly to the high burden and limited seasonality of *P. vivax* in PNG children [18,26]. A previous study in PNG children aged 1–5 y observed that presumptive artesunate (7 d) and PQ (14 d, partially supervised) mass treatment to clear hypnozoites reduced the risk of *P. vivax* clinical episodes by 28% ($p = 0.042$) compared to only blood-stage treatment and by 33% ($p = 0.015$) compared to no treatment [27]. Although the study used a suboptimal treatment regimen, and thus substantially underestimated the hypnozoite burden, it did highlight the significant challenge relapses pose to successful control and eventual elimination of *P. vivax* malaria in PNG and provides a strong rationale for conducting a more comprehensive clinical trial with an in-depth molecular diagnostics component.

Intensified national control efforts have seen the prevalence of light microscopy (LM)–detectable *P. vivax* malaria parasites in the general population decrease from 17% in 2006 to 8% in 2010 [25] and to 0.5% in 2014 [28]. Despite these gains, a large reservoir of individuals

infected with submicroscopic *P. vivax* persists. In a 2010 survey, *P. vivax* prevalence by qPCR was 12.8%. Of these infections, 89.6% were asymptomatic, 53.8% were submicroscopic, and 48.9% included *P. vivax* gametocytes [25]. Similarly high rates of persistent asymptomatic *P. vivax* infection and gametocyte carriage were also found in surveys in Thailand [29,30] and Brazil [31], which have seen substantial recent reductions in transmission.

Mass screening and treatment (MSAT) and mass drug administration (MDA) with artemisinin-based combination therapies have been advocated as important tools to reduce the asymptomatic *P. falciparum* reservoir [32,33]. These interventions are also likely to be of great importance for *P. vivax* elimination. Significant questions remain, however, none greater than how to best attack the undetectable hypnozoite reservoir.

To address these critical questions we have conducted a randomised double-blind placebo-controlled trial of a highly efficacious PQ treatment regimen in PNG children aged 5–10 y, using detailed molecular diagnostics to directly measure the contribution of relapses to the burden of *P. vivax* and *P. ovale* infection, disease, and transmission. By using these data in mathematical models, we further estimate the potential effect of MDA with treatment regimens that are part of first-line policy and regimens currently under investigation in clinical trials [34,35] on the prevention of recurrent *P. vivax* infections, providing critical evidence-based recommendations for policy-makers.

Methods

Ethics Statement

The protocol (S1 Text) received ethical clearance from the PNG Institute of Medical Research Institutional Review Board (0908), the PNG Medical Advisory Committee (09.11), and the Ethics Committee of Basel (237/11) and was conducted in full accordance with the Declaration of Helsinki. The study was retrospectively registered at ClinicalTrials.gov (NCT02143934) on 20 May 2014 due to the fact that it was designed and perceived by the investigating team as a treatment to re-infection cohort study where randomisation of PQ treatment was being done in order to modify exposure and allow a detailed investigation of relapses rather than as a trial to assess the efficacy of this specific treatment schedule. However, following further review, it was decided that the study did indeed fulfil the generally accepted definitions of a clinical trial, and the study was then retrospectively registered at ClinicalTrials.gov. All authors affirm that other trials involving PQ that they are involved in are registered at ClinicalTrials.gov (NCT01837992; NCT02364583).

Study Site, Design, and Participants

The study was conducted from 17 August 2009 to 20 May 2010 in five village clusters (13 hamlets) of the Albinama and Balif areas of Maprik District, East Sepik Province, PNG, where both *P. falciparum* and *P. vivax* are hyperendemic and *P. vivax* is responsible for the majority of malaria infection and disease in the first 3 y of life [18,22,36]. Malaria transmission is moderately seasonal, peaking in the early wet season from December to March [26]. Health services for the area are provided by the Albinama health sub-centre, the Balif aid post, and a system of village-based health workers operating in all study villages.

Between 17 August and 11 September 2009, 529 children aged 5 to 10 y whose parents provided written informed consent for their participation were screened for inclusion into this parallel double-blind placebo-controlled trial. Children were enrolled if they fulfilled the following criteria: (i) aged 5–10 y (± 3 mo in children without known date of birth, (ii) enrolled at selected elementary schools and permanent residents of the area, (iii) no disability, (iv) no chronic illness, (v) no known allergy to study drugs, (vi) haemoglobin > 50 g/l, (vii) no severe

malnutrition (defined by the PNG national guidelines as weight-for-age nutritional Z score < 60th percentile), and (viii) no G6PD deficiency. The inclusion criteria were amended during the study to allow children who were aged 5–6 y but who were not yet enrolled in elementary school to participate in the study. This was necessary to ensure we adequately covered the desired age range of 5–10 y and reached the required sample size of 525 without expanding the geographical area. Five children were excluded on the basis of G6PD deficiency, and 524 were block randomised using a 1:1 allocation ratio to receive 20 d of directly observed treatment (DOT) over 4 wk (26 d) of either (i) chloroquine (CQ) (DOTs 1–3), AL (Coartem) (DOTs 11–13), and PQ (DOTs 1–20; 0.5 mg/kg) or (ii) CQ (DOTs 1–3), AL (DOTs 11–13), and placebo (PL) (DOTs 1–20) (Fig 1). This treatment regimen was deliberately chosen to maximise efficacy, and the dose of each drug was timed such that there would be minimal residual drug by day 0 of the follow-up period (baseline). In order to achieve this, a 4-wk washout period was required for CQ, and the 20 d of PQ DOTs were scheduled Monday to Friday of these 4 wk (Fig 1), for ease of direct supervision of every dose. AL was administered because there is documented CQ resistance in PNG, and the PNG national treatment guidelines for *P. vivax* are to use AL plus PQ. The administration of AL on DOTs 11–13 (days 15–17) was deliberate so that it wouldn't interfere with CQ and so that drug levels would have reduced to zero by baseline. The intention was not to trial this unconventional drug regimen as a treatment for implementation, but rather to devise a maximally effective treatment to ensure radical cure in half of the cohort and thus allow a detailed investigation of relapses. Participants, field teams, and investigators were all blinded with respect to treatment allocation.

Randomisation, Blinding, and Treatment Allocation

After enrolment, children were randomly allocated to the PQ or PL treatment group using a pre-assigned list. Randomisation lists were prepared by an independent statistician using Microsoft Excel and consisted of ID assignments in blocks of six, each block comprising a list of the same six letters in random order. The independent statistician assigned three randomly selected letters to the PQ drug containers and the three other letters to the PL drug containers. The coding document was held by the statistician until completion of the trial. The PQ and PL tablets were identical in size, shape, and colour. The entire study team and principal investigators remained fully blinded for the entire study period.

Clinical Procedures and Follow-Up

Clinical assessment at enrolment included screening for symptoms of febrile illness, a detailed history of bednet use and recent illness/anti-malarial treatment, and collection of a finger-prick blood sample for assessment of G6PD deficiency using the visual, tube-based G6PD assay (Dojindo Laboratories, Japan), haemoglobin measurement, and later immunological and molecular studies. Children who were febrile were tested using a malaria rapid diagnostic test (RDT) (CareStart Malaria pLDH/HRP2 Combo; AccessBio, US); children with a positive test were treated with AL during DOTs 1–3. DOT 1 was administered at the end of the enrolment visit, with the subsequent 19 DOTs administered daily Monday–Friday over the subsequent 4 wk (Fig 1). All DOTs were supervised by a member of the clinical field team and were co-administered with food and well tolerated [37]. Safety monitoring was performed and reported previously [27]. No specific safety monitoring was conducted during post-intervention follow-up.

Three days after the final DOT (i.e., 4 wk after enrolment), a venous sample was collected and defined as baseline (time point day 0 of the follow-up period). Children were then actively monitored for the presence of febrile symptoms every fortnight for 8 mo. To ensure the capture

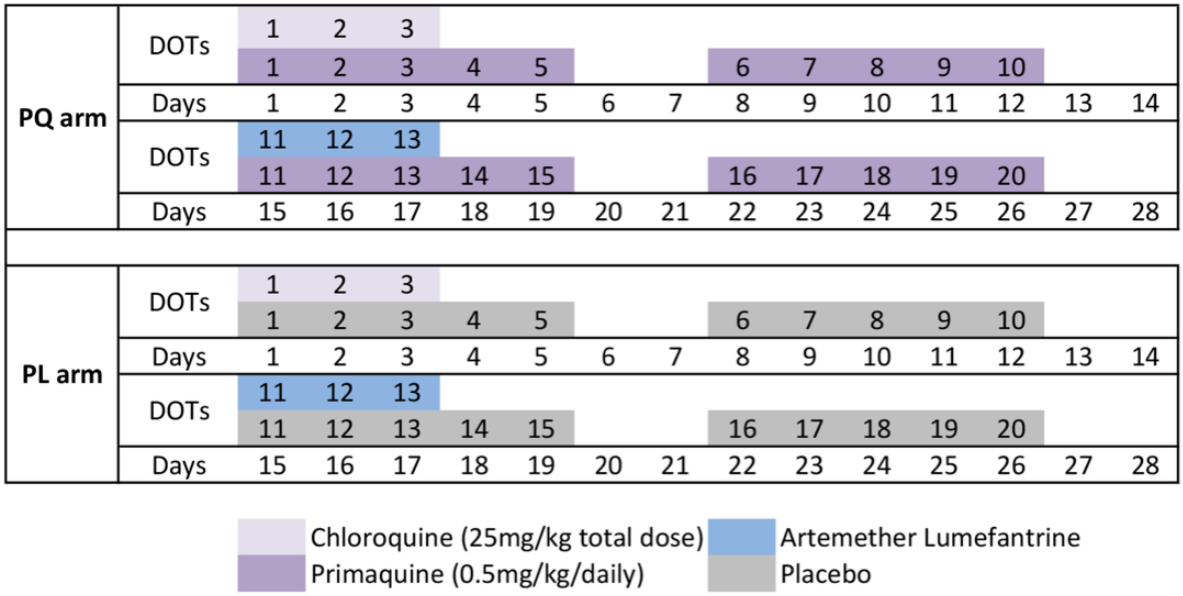

Fig 1. Drug administration schedule. The drug regimen for the PQ and PL treatment arms was administered with direct observation on 20 d (Monday to Friday) over a 4-wk (26-d) period.

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of information related to any episodes of illness in the 2 wk between visits, passive surveillance measures were implemented at local health centres and aid posts and via the village health worker network. In all symptomatic children, *Plasmodium* spp. infection was initially confirmed using RDT and a 250-μl finger-prick blood sample was collected for confirmation of infection by LM and qPCR. Only symptomatic children who tested positive by RDT and/or LM were treated with AL. PQ was not re-administered for RDT- or LM-confirmed *P. vivax* malaria episodes during follow-up. All other illness episodes detected were referred to the local health centre and treated in accordance with PNG treatment guidelines. Finger-prick blood samples were also collected from all children every 2 wk for the first 12 wk and every 4 wk thereafter during the follow-up period (Fig 2). Children were considered lost to follow-up if they permanently relocated outside of the study area or withdrew from the study.

Laboratory Methods

All blood samples collected during active and passive surveillance were examined by LM and qPCR. Blood films were examined independently by two skilled microscopists, blind to allocated treatment, for 200 thick-film fields (1,000× magnification) before being declared *Plasmodium*-negative. Parasite density was calculated from the number of parasites per 200–500 leucocytes (depending on parasite density) and an assumed leucocyte density of 8,000/μl [38]. Slides discrepant for positivity/negativity, species determination, or density (>2 log difference) were adjudicated by a WHO-certified level 1 (expert) microscopist. Slides were scored as LM-positive for an individual *Plasmodium* species if the species was detected independently by at least two microscopists and/or if subsequent qPCR diagnosis confirmed the presence of the species. Densities were calculated as the geometric mean densities of all positive reads.

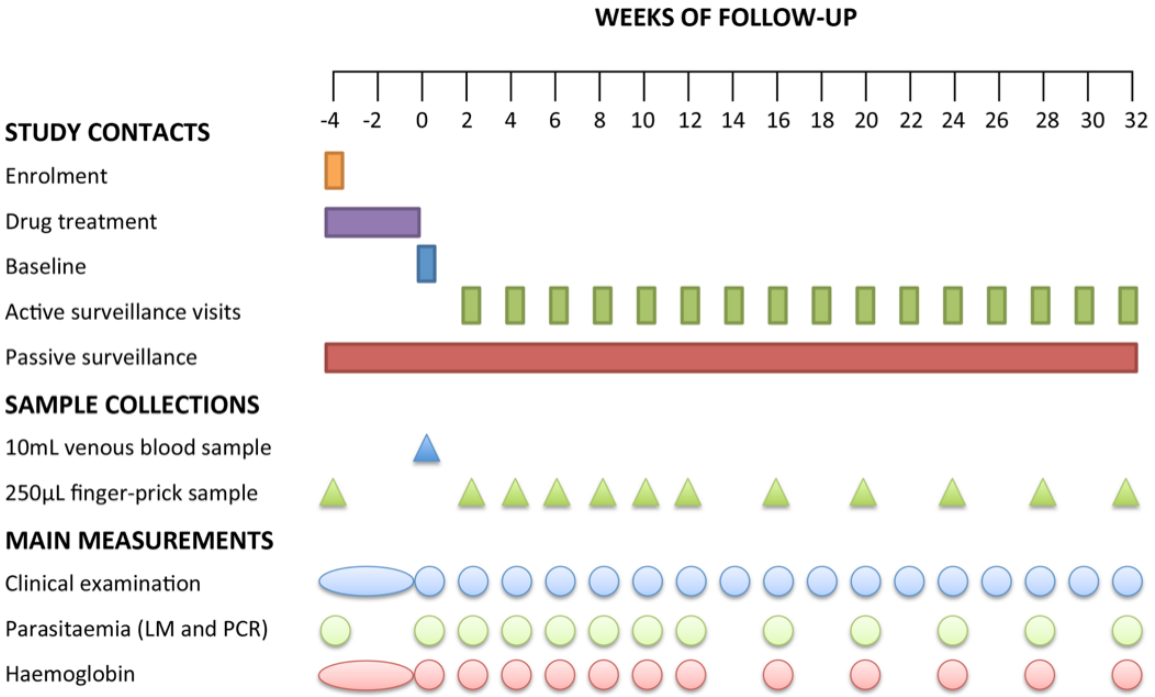

Fig 2. Study design and follow-up schedule. After the drug treatment period, children were actively monitored for infection and illness every 2 wk for the first 12 wk. From week 14 to 32, children were actively monitored for illness every 2 wk and for infection every 4 wk.

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Venous blood samples were separated into plasma, peripheral blood mononuclear cells and red cell pellets, which were stored at −80°C, in liquid nitrogen, and at −20°C, respectively. Finger-prick blood samples were separated into plasma and red cell pellets and stored at −80 and −20°C, respectively. DNA was extracted using the FavorPrep 96-Well Genomic DNA Extraction Kit (Favorgen, Taiwan) from the red cell pellet fraction of all samples. *Plasmodium* spp. infections were detected using a generic qPCR to detect all four species, after which species-specific (*P. falciparum*, *P. vivax*, *P. malariae*, and *P. ovale*) qPCRs were performed on *Plasmodium*-positive samples [39,40]. The *P. ovale* PCR detects both *P. ovale curtisi* and *P. ovale wallikeri*. In addition, samples positive for *P. vivax* by qPCR were tested for gametocytes by *Pvs25* quantitative reverse transcription PCR (qRT-PCR) [39], and individual *P. vivax* clones were genotyped by capillary electrophoresis using the molecular marker *msp1F3* [41] to determine the number of genetically distinct *P. vivax* blood-stage clones acquired per individual per year at risk, i.e., the molecular force of blood-stage infection ($_{mol}$FOB) [42].

Statistical Analysis and Modelling

As the primary objective of the study was to determine relapse frequencies, the analysis was by modified intention to treat, excluding children who received fewer than 14 DOTs but including those that received 14–20 DOTs and had incomplete follow-up. For analysis purposes, a clinical episode of *P. vivax* or *P. falciparum* malaria was defined as febrile illness (current or previous 48 h) plus the presence of *P. vivax* or *P. falciparum* parasites by LM (any density). The

primary trial endpoint was pre-defined as time to first *P. vivax* infection after baseline by qPCR. Secondary endpoints included time to first *P. vivax* infection after baseline by LM; time to first *P. vivax* clinical episode; time to first *P. vivax* gametocyte positivity; incidence rate of clinical *P. vivax* episodes; incidence rate of genetically distinct *P. vivax* infections ($_{mol}$FOB); incidence rate of *P. vivax* gametocyte positivity; time to first *P. falciparum*, *P. malariae*, and *P. ovale* infection by qPCR; and time to first *P. falciparum* clinical episode. A minimum sample size of 250 children per arm was calculated using hazard-rate-based calculations (log-rank tests) based on the effect (30% reduction in incidence risk in PQ group) observed in [27] with an α–error of 5% and a power of 80% and assuming a 30% increase in time to first infection following additional distributions of long-lasting insecticide-treated nets. Sample sizes were increased by 5% to account for children receiving fewer than 14 doses of PQ or PL.

Time to first *Plasmodium* infection or clinical episode and its association with treatment and covariates were modelled using Cox regression, and the proportional hazards (PH) assumption was checked using the test based on the Schoenfeld residuals. For these analyses, time at risk was censored on the last day before the first of two consecutively missed clinical follow-up visits, resulting in the censoring of 177 children (Fig 3). Kaplan-Meier estimates were computed for each endpoint by *Plasmodium* species and method of *Plasmodium* diagnosis. The log-rank test was used to test for differences between survival curves. In all survival analyses, children were considered "at risk" until they reached the endpoint of interest, withdrew, were lost to follow-up, were censored, or completed the study. Village membership was fitted as a fixed effect in the Cox PH model using the following equation:

$$h(t) \quad = h_0(t) \quad \exp(\beta_1 x_1 + \quad \beta_2 x_2 + \quad \beta_3 x_3 + \quad \beta_4 x_4) \qquad (1)$$

where x_1 = treatment, x_2 = age, x_3 = village, and x_4 = infection status by the same *Plasmodium* species at enrolment.

Negative binomial regression (NB reg) models were used to calculate the incidence rate of clinical episodes, $_{mol}$FOB, and *P. vivax* gametocyte positivity. In these models, time at risk was calculated for individual children based on the number of attended versus missed visits. If a child was not seen for a consecutive time period of 108 d during the follow-up period, time at risk was censored at the last attended follow-up visit or passive surveillance contact. This resulted in 74 children being censored (44 in PL arm and 30 in PQ arm) rather than the 177 shown in Fig 3. In addition, for the molecular analyses, time at risk was reduced for children who were not seen for consecutive intervals of 42 d by subtracting the days of the missed intervals from the overall individual time at risk. As per PNG treatment guidelines, treatment during the follow-up period was given only if a clinical episode was detected. A potential competing risk could exist if treatment was administered for a clinical episode with one species before the first event for a heterologous species endpoint had occurred. However, since the incidence rate of clinical episodes was very low, this occurred very rarely. It should also be noted that the times at risk for the analyses of different endpoints were not adjusted for the post-treatment prophylactic effects of the aforementioned treatments. Clinical malaria episode incidence rate ratios (IRRs) were derived from models adjusted for treatment, age, and *P. vivax* positivity by PCR at enrolment, while the models for $_{mol}$FOB and *P. vivax* gametocyte positivity were further adjusted for village of residence. Differences between treatment groups at enrolment were investigated using chi-square and Fisher's exact tests for categorical characteristics and Student's *t*-test for normally distributed continuous variables. All tests were two-tailed, and the confidence level was set at 95%. All analyses were performed using R version 3.0.3. [43] and/or Stata version 12 [44].

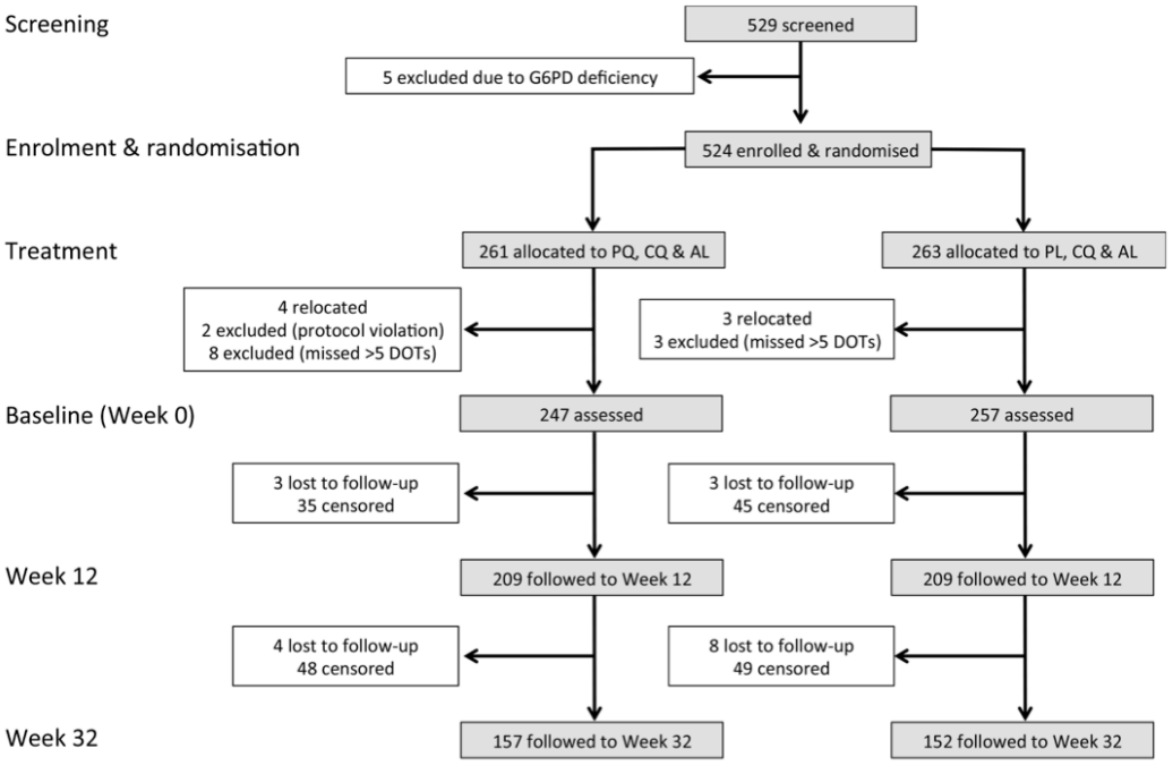

Fig 3. Consort diagram: study design, randomisation, and retention of study participants during follow-up. In the analysis of time to first infection and clinical episode, children were censored on the last day before the first of two consecutive missed clinical visits.

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The effects of MDA and MSAT programmes with anti-malarial drugs currently recommended as first-line treatment on the dynamics of *P. vivax* and *P. falciparum* transmission were investigated using a mathematical model. The model simulated the impact of a 3-d course of either dihydroartemisinin-piperaquine (DHA-PIP) or CQ to clear blood stages [45], and a 14-d PQ regimen to clear liver stages. The model also simulated the impact of tafenoquine plus CQ treatment, a highly promising short-course anti-relapse therapy that is currently undergoing phase 3 clinical trials [34]. A classical Ross-Macdonald model [46,47] was used to describe the qualitative dynamics of *P. falciparum* following treatment of "at risk" populations with drugs for clearing blood-stage infections. This model was extended to incorporate relapse infections of *P. vivax* and the effects of PQ treatment for the clearance of liver-stage hypnozoites [48]. In brief, individuals in a population can be susceptible to blood-stage infection with no liver-stage hypnozoites (S_0), infected with blood-stage parasites but no hypnozoites (I_0), susceptible to blood-stage infection but carrying hypnozoites (S_L), or infected with both blood-stage parasites and hypnozoites (I_L). Full details of the deterministic differential equations describing the mathematical model and parameter definitions are provided in S1 Table. The model was also implemented in a stochastic framework with population size 5,000 to capture stochastic variation and the potential for the elimination of transmission.

Details of all methods used in this study are given in S1–S3 Text, S1 Table, and S1 and S2 Figs, and all datasets are available in the Dryad repository: http://dx.doi.org/10.5061/dryad.m1n03 [49].

Results

Enrolment and Baseline Characteristics of Children

Of the 524 G6PD-normal children who were randomised to receive PQ/CQ/AL (PQ arm) or PL/CQ/AL (PL arm), a total of 504 children aged 4.8–10.5 y completed at least 14 d of DOT with PQ or PL and remained in the study at baseline (day 0 of follow-up) and were thus actively and passively monitored for 32 wk post-baseline (Fig 3).

No significant differences in demographic characteristics or infection status were observed at enrolment between the treatment groups (Table 1). At baseline (~4 wk after enrolment and commencement of drug treatment), four children were *P. vivax*–positive by qPCR (two each in the PL and PQ arms), two children in the PL arm were *P. falciparum*–positive, and one of these was also *P. vivax*–positive. These children therefore did not contribute to time at risk and were thus excluded from the respective analyses of time to first infection. No *Plasmodium* infections were detected by LM at baseline. During follow-up, 81.6% (2,308/2,827) of scheduled visits were attended by children in the PL arm, and 82.7% (2,248/2,717) in the PQ arm. The average number of all study contacts during follow-up did not differ between the treatment arms (PL arm: 14.0, PQ arm: 14.4, Student's *t*-test, $p = 0.25$).

Risk of *P. vivax* Infection during Follow-Up

The time to first or only *P. vivax* blood-stage infection (as detected by qPCR) differed significantly between the two treatment arms: only 25.5% (63/247) of children who received PQ experienced at least one new *P. vivax* infection, compared to 65.0% (167/257) in the PL arm (Cox PH, $p < 0.001$; Table 2; Fig 4A). qPCR-positive, recurrent *P. vivax* blood-stage infections were detected rapidly in the PL arm, with 31.5% (80/254) of children infected by day 42 compared to only 8.2% (20/245) in the PQ arm (Fig 4A). As expected, LM-positive infections were less common in both arms and were observed later during follow-up (Fig 4B).

Clearance of liver stages through PQ treatment resulted in an 82%–84% reduction in the risk of recurrent *P. vivax* blood-stage infections diagnosed by qPCR (hazard ratio [HR] = 0.18 [95% CI 0.14, 0.25], Cox PH, $p < 0.001$) and LM (HR = 0.16 [95% CI 0.11, 0.24], Cox PH, $p < 0.001$) compared to PL (Table 2). This increased to an 83%–88% reduction in risk when only the first 3 mo of follow-up were considered (qPCR: HR = 0.17 [95% CI 0.12, 0.24], Cox PH, $p < 0.001$; LM: HR = 0.12 [95% CI 0.07, 0.19], Cox PH, $p < 0.001$; Table 2).

There was considerable heterogeneity in the prevalence and risk of *P. vivax* infection across the study villages. The risk of first *P. vivax* infection (by qPCR and LM) differed significantly among children living in different villages, with the highest risk in Bolumita (qPCR: HR = 4.70 [95% CI 3.14, 7.02], Cox PH, $p < 0.001$; LM: HR = 4.62 [95% CI 2.92, 7.30], Cox PH, $p < 0.001$, reference village Albinama) and Balanga (qPCR: HR = 2.33 [95% CI 1.53, 3.55], Cox PH, $p < 0.001$; LM: HR = 1.70 [95% CI 1.03, 2.81], Cox PH, $p = 0.04$, reference village Albinama). There was, however, no interaction between village and treatment effect. The risk of first *P. vivax* blood-stage infection diagnosed by qPCR after treatment was not significantly associated with age (qPCR: HR = 0.95 [95% CI 0.87, 1.03], Cox PH, $p = 0.19$); however, there was a 15% reduction in the risk of having at least one LM-patent *P. vivax* infection with each additional year of life (LM: HR = 0.85 [95% CI 0.77, 0.95], Cox PH, $p = 0.003$). As with village, there was no interaction between age and treatment effect.

Table 1. Demographic and clinical characteristics of the cohort at enrolment, classified by allocated treatment.

Characteristic	Total (*n* = 504)	PL Arm (*n* = 257)	PQ Arm (*n* = 247)	*p*-Value
Male sex	49.2%	49.8%	48.6%	0.78
Age (years)	7.6 ± 1.6	7.7 ± 1.5	7.5 ± 1.6	0.40
Weight (kilogrammes)	19.7 ± 3.4	19.7 ± 3.2	19.8 ± 3.6	0.82
Village				0.57
Albinama	22.8%	23.0%	22.7%	
Amahup	26.0%	27.3%	24.7%	
Balanga	10.9%	12.5%	9.3%	
Balif	25.6%	24.5%	26.7%	
Bolumita	14.7%	12.8%	16.6%	
Bednet use	93.3%	93.4%	93.1%	0.91
Infection by qPCR*				
P. vivax	47.4%	44.8%	50.2%	0.22
P. falciparum	23.8%	23.7%	23.9%	0.97
P. ovale	3.4%	1.9%	4.9%	0.07
P. malariae	14.3%	13.2%	15.4%	0.49
Non–*P. vivax*	12.9%	14.0%	11.7%	0.45
Fever	14.9%	15.2%	14.6%	0.85
Haemoglobin (g/l)	108 ± 13	109 ± 13	108 ± 13	0.78

Data are percentage or mean ± standard deviation. Analysis is by chi-square and Fisher's exact tests for categorical characteristics and Student's *t*-test for normally distributed continuous variables.

*Prevalence includes single- and mixed-species infections.

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When all *P. vivax* infections in a child were genotyped to identify new infections in the context of ongoing multiple clone infections, PQ treatment was associated with a 77% reduction in the incidence of genetically distinct *P. vivax* blood-stage clones ($_{mol}$FOB) compared to PL (PQ arm: $_{mol}$FOB = 1.62/y, PL arm: $_{mol}$FOB = 4.74/y, IRR = 0.23 [95% CI 0.18, 0.31], NB reg, $p < 0.001$; Table 3). The effect of the PQ treatment on $_{mol}$FOB was substantially larger in the first 3 mo (IRR = 0.21 [95% CI 0.15, 0.28], $p < 0.001$) than in months 4–8 (IRR = 0.34 [95% CI 0.24, 0.48], NB reg, $p < 0.001$; Table 3).

In addition, PQ treatment was associated with a 75% reduction in the hazard of becoming positive for *P. vivax* gametocytes by *Pvs25* qRT-PCR compared to PL (HR = 0.25 [95% CI 0.17, 0.37], Cox PH, $p < 0.001$; Table 2). The incidence rate of *P. vivax* gametocytes (defined as the number of samples positive for *Pvs25* qRT-PCR during follow-up) was more strongly reduced in the PQ arm compared to the PL arm in the first 3 mo (IRR = 0.18 [95% CI 0.11, 0.30], $p < 0.001$) than in the subsequent 5 mo (months 4–8: IRR = 0.37 [95% CI 0.24, 0.57], NB reg, $p < 0.001$; Table 3).

Irrespective of treatment group, clinical *P. vivax* malaria episodes were rare, with only 28 children experiencing one or more clinical *P. vivax* episodes during the 32-wk follow-up period (incidence risk: 0.19/child/y in PL arm and 0.06/child/y in PQ arm; Table 2). The clearance of hypnozoites by PQ treatment was associated with an 81% reduction compared to PL in the hazard of experiencing a *P. vivax* clinical episode of any density in the first 3 mo of follow-up (HR = 0.19 [95% CI 0.06, 0.58], Cox PH, $p = 0.004$; Table 2; Fig 4C) and a 75% reduction in the entire follow-up period (HR = 0.25 [95% CI 0.11, 0.61], Cox PH, $p = 0.002$; Table 2).

Table 2. Incidence of first (or only) *P. vivax*/*P. ovale* re-infections, *P. vivax* clinical malaria episodes, and *P. vivax* gametocyte positivity in treatment groups during the entire 8-mo follow-up period and during the first 3 mo of follow-up.

Outcome	PL Arm			PQ Arm			Cox Model Estimate*	
	Number of Events	PYR	Incidence Risk	Number of Events	PYR	Incidence Risk	HR (95% CI)	*p*-Value
***P. vivax* infections by qPCR**								
Months 0–8	167	63.6	2.62	63	100.6	0.63	0.18 (0.14, 0.25)	<0.001
Months 0–3	152	43.4	3.50	48	54.9	0.87	0.17 (0.12, 0.24)	<0.001
***P. vivax* infections by LM**								
Months 0–8	122	83.4	1.46	40	111.7	0.36	0.16 (0.11, 0.24)	<0.001
Months 0–3	104	51.3	2.03	22	58.3	0.38	0.12 (0.07, 0.19)	<0.001
***P. vivax* clinical malaria episodes**								
Months 0–8	21	109.0	0.19	7	112.5	0.06	0.25 (0.11, 0.61)	0.002
Months 0–3	15	61.2	0.25	4	60.2	0.07	0.19 (0.06, 0.58)	0.004
***P. vivax* gametocyte positivity**								
Months 0–8	99	93.3	1.06	35	112.9	0.31	0.25 (0.17, 0.37)	<0.001
Months 0–3	70	53.9	1.30	16	58.9	0.27	0.18 (0.10, 0.30)	<0.001
***P. ovale* infections by qPCR**								
Months 0–8	17	118.0	0.14	7	120.6	0.06	0.31 (0.13, 0.77)	0.011
Months 0–3	10	61.7	0.16	1	60.3	0.02	0.08 (0.01, 0.67)	0.019

**P. vivax* Cox PH regression model estimates with adjustment for treatment, age, village, and *P. vivax* infection status at enrolment. *P. ovale* Cox PH regression model estimates adjusted for treatment, age, village, and *P. ovale* infection status at enrolment. The number of children considered to be at risk on day 0 was 257 in the PL arm and 247 in the PQ arm, except for *P. vivax* infections by PCR, where corresponding numbers at risk were 254 and 245, respectively, since infections on day 0 were considered treatment failures and hence excluded.

HR, hazard ratio; PYR, person-years at risk.

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Risk of Non–*P. vivax* Malaria Infection during Follow-Up

Although the number of *P. ovale* infections diagnosed by PCR in either arm was low (incidence risk: both arms: 0.10 infections/child/y, PQ arm: 0.06, PL arm: 0.14), PQ treatment was associated with a 92% reduction compared to PL in the risk of *P. ovale* blood-stage infection diagnosed by qPCR in the first 3 mo of follow-up (HR = 0.08 [95% CI 0.01, 0.67], Cox PH, $p = 0.019$) and a 69% reduction in the entire 8-mo of follow-up period (HR = 0.31 [95% CI 0.13, 0.77], Cox PH, $p = 0.011$; Fig 4F; Table 2).

There was no significant association between PQ treatment and time to first (or only) *P. falciparum* blood-stage infection by qPCR (Cox PH, $p = 0.104$; Fig 4D; Table 4) or LM (Cox PH, $p = 0.706$; Table 4), and no effect of PQ treatment on time to first (or only) *P. falciparum* clinical episode during the 8 mo of follow-up (Cox PH, $p = 0.333$; Table 4). There was no significant association between PQ treatment and time to first (or only) *P. malariae* blood-stage infection by qPCR in the first 3 mo of follow-up (HR = 0.36 [95% CI 0.13, 1.02], Cox PH, $p = 0.055$) or during the entire follow-up period (HR = 0.55 [95% CI 0.24, 1.26], Cox PH, $p = 0.157$; Fig 4E; Table 4).

Implications for Malaria Control and Elimination Strategies

MSAT interventions target only individuals with detectable blood-stage parasitaemia. In the current cohort, 47.4% (239/504) of children had a *P. vivax* infection (single or mixed species) at enrolment, and another 12.9% (65/504) were positive with non–*P. vivax* blood-stage infections (Table 1). In the absence of PQ treatment, children with no patent infections at enrolment were significantly less rapidly re-infected with *P. vivax* (55.2% [58/105] became infected during 8 mo of follow-up) than those that had patent *P. vivax* (70.5% [79/112]) or *P. falciparum*/*P.*

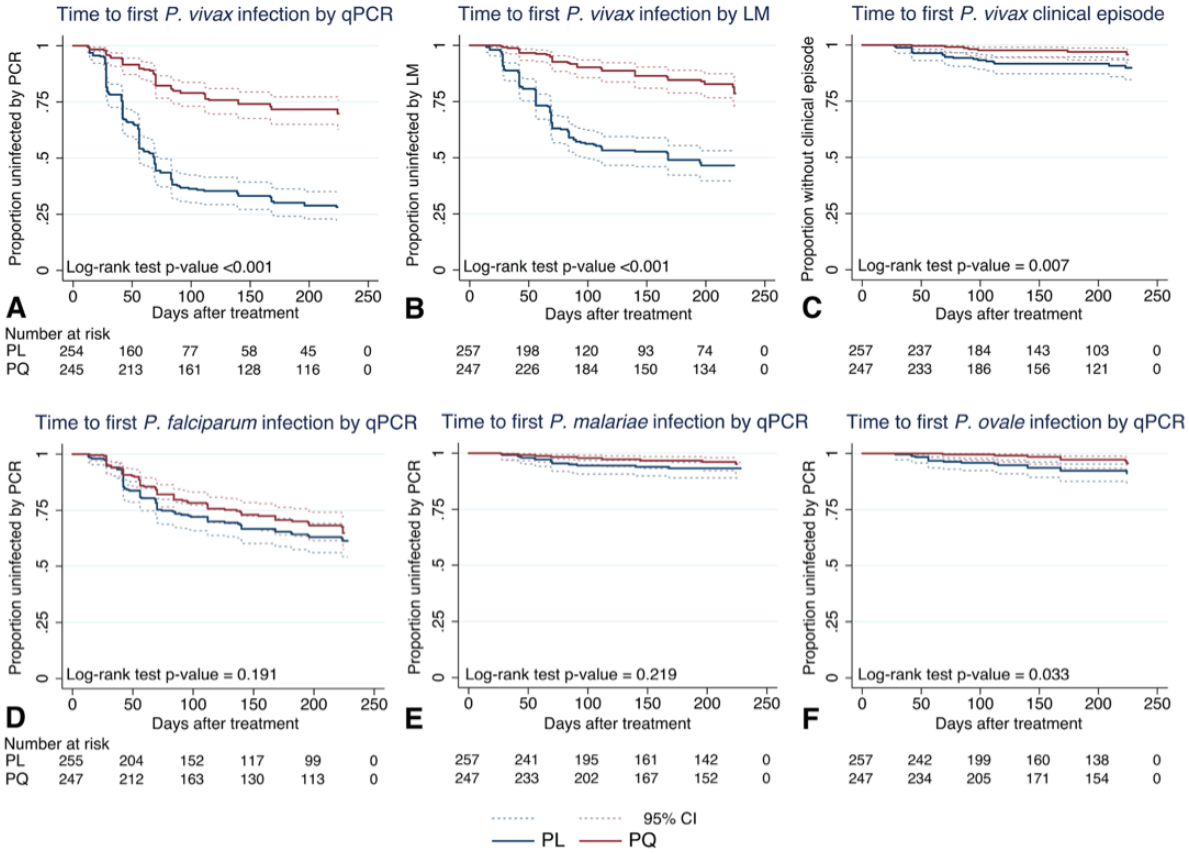

Fig 4. Time to first *Plasmodium* spp. infection and *P. vivax* clinical episode. Kaplan-Meier plots showing the time to first (or only) (A) *P. vivax* infection by qPCR, (B) *P. vivax* infection by LM, (C) *P. vivax* clinical episode, (D) *P. falciparum* infection by qPCR, (E) *P. malariae* infection by qPCR, and (F) *P. ovale* infection by qPCR, in the two treatment arms. Dashed lines represent the respective 95% confidence intervals.

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malariae/*P. ovale* infections (81.1% [30/37], log-rank test, $p < 0.001$; Fig 5; Table 5). In the PQ group, children with no patent infections were also less likely to be re-infected (17.9% [17/95]) than those in the other two groups (*P. vivax* patent infection: 31.7% [39/123], non–*P. vivax* patent infection: 25.9% [7/27], log-rank test, $p = 0.0412$; Fig 3; Table 5), indicating that children with no patent infections were more likely to live in low-transmission areas. PQ treatment was therefore equally efficient in preventing recurrent *P. vivax* infections in children with *P. vivax*, non–*P. vivax*, or no blood-stage infection at enrolment (Cox regression, adjusted for age and village of residence: likelihood ratio = 3.87, df = 2, $p = 0.14$ for treatment-by-infection status interaction).

The potential effect of MDA and MSAT with either blood-stage drugs only or blood- plus liver-stage drugs on the population prevalence of *P. vivax* and *P. falciparum* infections was further investigated using a basic stochastic transmission model. Administration of blood-stage drugs (DHA-PIP or CQ) was assumed to clear existing blood-stage infections and provide a 4-wk period of prophylactic protection against new infections, tafenoquine plus CQ was

Table 3. Incidence rate of *P. vivax* clinical malaria of any density, genetically distinct *P. vivax* blood-stage clones, and gametocyte positivity in treatment groups during the entire 8-mo follow-up period and separately for months 0–3 and 4–8 of follow-up.

Outcome	PL Arm			PQ Arm			Model Estimate*	
	Number of Events	PYR	Incidence Rate	Number of Events	PYR	Incidence Rate	IRR (95% CI)	*p*-Value
***P. vivax* clinical malaria episode—any density**								
Months 0–8	23	139.8	0.16	11	139.6	0.08	0.43 (0.21, 0.90)	0.026
Months 0–3	15	55.9	0.30	5	54.3	0.09	0.31 (0.11, 0.86)	0.025
Months 4–8	7	83.0	0.08	5	83.1	0.06	0.59 (0.17, 2.08)	0.410
Genetically distinct *P. vivax* blood-stage clones ($_{mol}$FOB)								
Months 0–8	653	122.1	4.74	196	120.6	1.62	0.23 (0.18, 0.31)	<0.001
Months 0–3	430	55.5	7.75	102	53.7	1.90	0.21 (0.15, 0.28)	<0.001
Months 4–8	221	66.5	3.32	94	66.0	1.42	0.34 (0.24, 0.48)	<0.001
***P. vivax* gametocyte positivity**								
Months 0–8	202	122.1	1.65	63	120.6	0.52	0.27 (0.19, 0.38)	<0.001
Months 0–3	101	55.5	1.82	20	53.7	0.37	0.18 (0.11, 0.30)	<0.001
Months 4–8	100	66.5	1.50	42	66.0	0.64	0.37 (0.24, 0.57)	<0.001

*The time at risk is calculated independently for each individual for each time range, with applicable study censoring for either clinical or PCR detection. Clinical malaria episode IRRs are derived from a NB reg adjusted for treatment, age, and *P. vivax* positivity by PCR at enrolment. Other IRRs are derived from a NB reg model adjusted for treatment, age, village, and *P. vivax* positivity by PCR at enrolment. The number of children considered at risk on day 0 was 245 in the PL arm and 253 in the PQ arm. The number of children considered at risk on study day 96 was 207 in the PQ arm and 205 in the PL arm.
PYR, person-years at risk.

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assumed to clear blood- and liver-stage infections and provide 8 wk of causal prophylactic protection (for full model details please refer to S1 Table). Fig 6 illustrates the predicted qualitative dynamics of *P. falciparum* and *P. vivax* transmission following two rounds of MDA (Fig 6A and 6B) or MSAT (Fig 6C and 6D) interventions with 80% coverage, separated by 6 mo. The interventions are predicted to cause a sharp decline in prevalence, followed by a gradual return to pre-intervention levels.

MDA is predicted to achieve much larger reductions in prevalence than MSAT, mostly due to the proportion of infections missed by the MSAT programme because of imperfect diagnostic sensitivity, but also because of the prophylactic protection in treated but uninfected individuals under the MDA programme.

Table 4. Incidence of first (or only) *P. falciparum*/*P. malariae* re-infection and *P. falciparum* clinical malaria in treatment groups in the entire follow-up period.

Outcome (Months 0–8)	PL Arm			PQ Arm			Cox Model Estimate*	
	Number of Events	PYR	Incidence Risk	Number of Events	PYR	Incidence Risk	HR (95% CI)	*p*-Value
P. falciparum infections by PCR	85	96.0	0.88	72	101.0	0.71	0.77 (0.56, 1.06)	0.104
P. falciparum infections by LM	52	108.8	0.48	53	107.1	0.50	0.93 (0.63, 1.37)	0.706
P. falciparum clinical malaria episodes	21	109.4	0.19	27	105.4	0.26	1.33 (0.75, 2.36)	0.333
P. malariae infections by PCR	15	118.2	0.13	9	119.4	0.07	0.55 (0.24, 1.26)	0.157

**P. falciparum* Cox PH regression model estimates adjusted for treatment, age, village, and *P. falciparum* infection status at enrolment. *P. malariae* Cox PH regression model estimates adjusted for treatment, age, village, and *P. malariae* infection status at enrolment. The number of children considered to be at risk on day 0 was 257 in the PL arm and 247 in the PQ arm, except for *P. falciparum* infections by PCR, where corresponding numbers at risk were 255 and 247, respectively, since infections on day 0 were considered treatment failures and hence excluded.
PYR, person-years at risk.

doi:10.1371/journal.pmed.1001891.t004

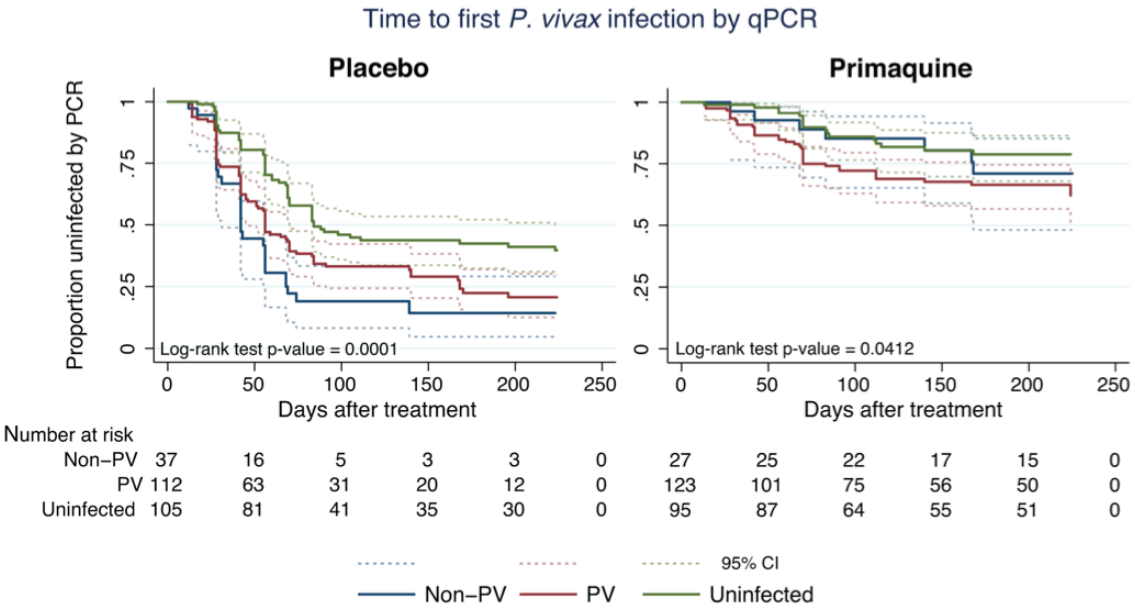

Fig 5. Kaplan-Meier plots showing the time to first (or only) *P. vivax* infection by qPCR in the PL and PQ arms, stratified by *Plasmodium* infection status at enrolment. Dashed lines represent the respective 95% confidence intervals. PV, *P. vivax*.

doi:10.1371/journal.pmed.1001891.g005

The initial reductions achieved by each of the interventions against *P. falciparum* and *P. vivax* are the same; however, when only blood-stage drugs are administered, a rapid rebound in *P. vivax* prevalence is predicted due to the hypnozoite reservoir, which is left unaffected by the treatment. Notably, *P. vivax* levels are predicted to return to pre-intervention levels within 6 mo post-intervention for both the MDA and MSAT interventions (blue curves in Fig 6A and

Table 5. *P. vivax* re-infection by qPCR in treatment groups during the entire 8 mo follow-up period and during the first 3 mo of follow-up, stratified by *P. vivax* infection status at enrolment.

Infection Status at Enrolment	PL Arm			PQ Arm			Cox Model Estimate*	
	Number of Events	PYR	Incidence Risk	Number of Events	PYR	Incidence Risk	HR (95% CI)	*p*-Value
Non–*P. vivax* infection (*n* = 65)								
Months 0–8	30	6.3	4.78	7	12.7	0.55	0.11 (0.04, 0.26)	<0.001
Months 0–3	29	5.1	5.73	4	6.6	0.61	0.08 (0.03, 0.24)	<0.001
***P. vivax* infection (*n* = 239)**								
Months 0–8	79	24.9	3.18	39	46.4	0.84	0.21 (0.14, 0.32)	<0.001
Months 0–3	71	18.0	3.95	32	26.3	1.22	0.21 (0.14, 0.33)	<0.001
Uninfected (*n* = 200)								
Months 0–8	58	32.4	1.79	17	41.4	0.41	0.19 (0.11, 0.34)	<0.001
Months 0–3	52	20.4	2.55	12	22.0	0.55	0.15 (0.08, 0.29)	<0.001

*Cox PH regression model estimates adjusted for treatment, age, village, and *P. vivax* infection status at enrolment. The number of children considered to be at risk on day 0 was 254 in the PL arm and 245 in the PQ arm.

PYR, person-years at risk.

doi:10.1371/journal.pmed.1001891.t005

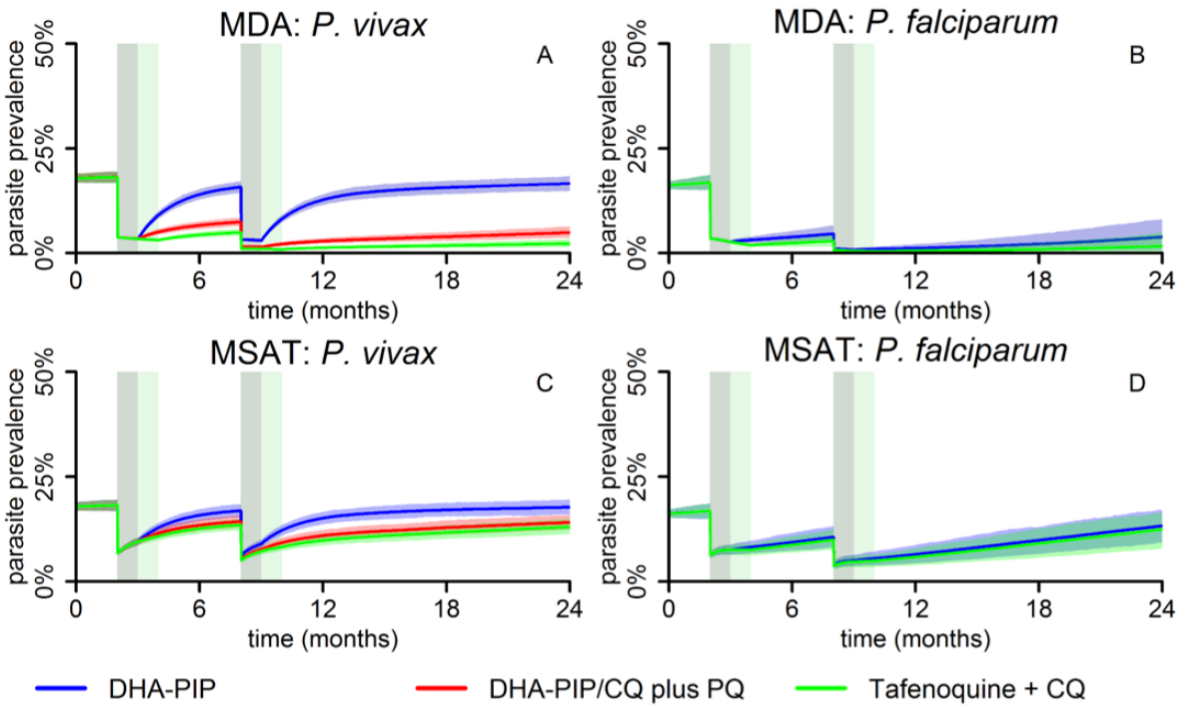

Fig 6. Mathematical-model-based predictions of impact of MDA and MSAT with either blood-stage drugs only or blood- plus liver-stage drugs on the population prevalence of *P. vivax* and *P. falciparum* infections. The effect of two rounds (6 mo apart) of MDA (A and B) or MSAT (C and D) with antimalarial drugs at 80% coverage on *P. vivax* (A and C) and *P. falciparum* (B and D) blood-stage parasite prevalence, as predicted by a stochastic model in a human population of size 5,000. The lines represent the mean of 1,000 repeat simulations, and the shaded areas represent the envelopes containing 95% of stochastic simulations. The grey and green shaded bars denote the duration of prophylactic protection for DHA-PIP/CQ and tafenoquine, respectively, after each treatment round. DHA-PIP and CQ were assumed to be administered as part of a 3-d regimen, providing prophylaxis for 1 mo. PQ was assumed to be administered as part of a 14-d regimen, providing prophylaxis for 15 d. Tafenoquine was assumed to be administered via a single dose, providing prophylaxis for 2 mo.

doi:10.1371/journal.pmed.1001891.g006

6C), in contrast to *P. falciparum*, where, following an MDA programme, prevalence is predicted to remain below pre-intervention levels for a sustained period of time (Fig 6B).

In the stochastic transmission model, only the addition of 8-aminoquinolines to a regimen of blood-stage drugs targets the hypnozoite reservoir and prevents the rapid resurgence of *P. vivax* blood-stage infections caused by relapses. Consequently, interventions with PQ are shown to result in a sustained reduction of the *P. vivax* burden (red curves in Fig 6A and 6C). Especially in the case of MDA (red curve, Fig 6A), *P. vivax* parasite prevalence is predicted to remain very low (<10%) during the modelled 16 mo post-intervention. Tafenoquine is estimated to be more effective than PQ at reducing prevalence because of the higher level of efficacy and the longer duration of causal prophylaxis [34].

Discussion

By randomising children to receive either a blood- and liver-stage treatment of PQ/CQ/AL (PQ arm) or a blood-stage only treatment of PL/CQ/AL (PL arm), and thereby selectively removing hypnozoites from half of the children in the cohort, this study confirms that relapses

from long-lasting liver stages account for four out of five *P. vivax* infections and three out of five *P. ovale* infections in PNG children aged 5–10 y living in an area with hyperendemic transmission. A transmission model estimated that MSAT with blood- and liver-stage treatment or MDA with blood-stage treatment alone would have only a transient effect on *P. vivax* transmission levels, while MDA with blood- and liver-stage treatment is predicted to be a highly effective strategy for *P. vivax* elimination.

The risk of having at least one PCR-detectable *P. vivax* infection during 8 mo of follow-up was reduced by 82% by PQ treatment. This reduction is substantially larger than the 44% reduction observed in an earlier study [27] that used a 30% lower dose of PQ combined with 7 d of artesunate, confirming that this earlier treatment regimen was not fully effective in preventing relapses. PQ also reduced the incidence of genetically distinct blood-stage infections ($_{mol}$FOB) [42] in the first 3 mo of follow-up by a similar amount (79%), indicating that relapses accounted for ~4/5 of all *P. vivax* infections in PNG children. Although the effect of the PQ treatment decreased with time since treatment, the incidence of new infections in the PQ arm was nevertheless still reduced by more than half (66%) after 3 mo of follow–up. This "washout" of the PQ effect is likely due to relapses activating rapidly in PNG [7] and the hypnozoite reservoir being replenished through continued exposure to new infected mosquito bites. The relatively sustained effect of the PQ treatment does however indicate that even in tropical *P. vivax* strains, a substantial proportion of hypnozoites remain dormant for three or more months.

Children who were not treated with PQ were also approximately four times more often positive for *P. vivax* gametocytes than children who were treated with PQ. Since even low-density *P. vivax* infections can infect mosquitoes [50–52], it is thus likely that relapsing infections are the primary source of *P. vivax* transmission.

PNG children acquire immunity to *P. vivax* rapidly [18], and, as shown in earlier studies in the same age group [20], clinical *P. vivax* episodes are rare. Nevertheless, PQ reduced the incidence of clinical *P. vivax* episodes by 69% in the first 3 mo after treatment, indicating that relapses can cause clinical episodes even in individuals with moderate levels of acquired immunity. Several studies have shown that relapses are often genetically distinct from the initial clinical episode [53,54]. These genetically diverse relapses may either be meiotic siblings [55] or originate from a previous (mosquito) bite and are likely to result in a higher risk of clinical illness in relapsing infection than that seen in malaria therapy patients infected twice with the same strain [10].

PQ treatment reduced the risk of PCR-detectable *P. ovale* infections by 92% in the first 3 mo of follow-up and by 69% in the entire follow-up period. This confirms that relapses account for a considerable portion of infections from this less prevalent species of relapsing malaria, and likely also sustain *P. ovale* transmission. This is relevant not only in PNG, but also in sub-Saharan Africa, where *P. ovale* prevalence can reach 4%–10% (by LM) in areas of west and central Africa [56]. We observed no significant association of PQ treatment with risk of PCR-detectable infections from the non-relapsing species *P. falciparum*. In the PQ arm, there was a non-significant ($p = 0.055$; only 24 total events) reduction in the risk of *P. malariae* infections in the first 3 mo of follow-up compared to the PL arm. Although *P. malariae* is not thought to form hypnozoites in the liver, chronic or relapsing infections of *P. malariae* up to 20 y after a person has left an endemic area have been documented [57,58]. Although it is currently not known how and where *P. malariae* infections can remain dormant for such extensive periods of time, our data suggest that such longer-lived *P. malariae* stages may be susceptible to PQ treatment. Larger clinical trials involving both symptomatic and asymptomatic *P. malariae* infections would be required to confirm the prevention of recurrent *P. malariae* infections by PQ treatment.

Given the very large contribution of relapses to the burden of *P. vivax* and *P. ovale* infection, illness, and transmission, it is essential that all *P. vivax*– and *P. ovale*–infected individuals receive therapy that is effective against both blood stages and relapses. PNG national standard treatment guidelines recommend treating confirmed *P. vivax* or *P. ovale* cases with 0.25 mg/kg PQ for 14 d; however, this recommendation is not being consistently implemented due to lack of access to point-of-care tests to screen for G6PD deficiency.

Interestingly, PQ was not only effective in preventing recurrent *P. vivax* infection in children who had PCR-detectable *P. vivax* infections but was equally effective in children with non–*P. vivax* infections and even those without any *Plasmodium* spp. infections at enrolment. All currently available malaria diagnostic tests identify only active blood-stage infections. They can thus not identify people who have *P. vivax* hypnozoites in their livers but are free of blood-stage infection. The rapid appearance of *P. vivax* infections and significant reduction in rates of recurrent infections in PQ-treated qPCR-negative children indicate that the number of PNG children without blood-stage infections who carry *P. vivax* hypnozoites is comparable to the number of children with *P. vivax* infections. The presence of active *P. vivax* blood-stage infection is thus a poor predictor for the risk of *P. vivax* relapse.

The rapid recurrence of *P. vivax* infections after treatment of *P. falciparum* infections has been observed in numerous *P. falciparum* in vivo drug efficacy trials, both in PNG [45,59] and elsewhere [60]. It has therefore been argued that anti-relapse therapy should be administered for all patients with malaria in regions of *P. vivax* co-endemicity [60]. Our results clearly support this recommendation and also extend it to asymptomatic parasite carriers.

With the renewed drive to eliminate malaria, the role of asymptomatic and/or submicroscopic infections in sustaining malaria transmission has become a major focus [15,61,62]. An increasing number of studies show that these infections contribute significantly to both *P. falciparum* and *P. vivax* transmission at all levels of endemicity [50–52,63,64]. As first noted by Robert Koch in 1900 during studies in then German New Guinea, the control of these infections is essential if elimination is to be achieved rapidly [65].

MSAT and MDA with artemisinin-based combination therapies are two interventions aimed at reducing the asymptomatic reservoir [32,66,67]. Although the simple model implemented in the present study agrees with the findings from more detailed *P. falciparum* models [67] that an MSAT programme with a highly sensitive diagnostic test such as qPCR can effectively reduce *P. falciparum* transmission, field trials have shown that MSAT with a less sensitive diagnostic test such as an RDT has limited or no effect on transmission [68–70]. As mass screening by PCR is difficult to implement, focalised MDA may be a more practical approach [71].

Our modelling predicted that MSAT will have only limited effectiveness for *P. vivax* even if conducted with a sensitive molecular diagnostic test and including an anti-liver-stage treatment since it will not target the blood-stage-negative population harbouring hypnozoites. MDA, on the other hand, is predicted to be highly effective in reducing the burden of future *P. vivax* infections but only if conducted with anti-blood- and anti-liver-stage treatment. Thus, effective control of *P. vivax* with anti-malarial drugs will require the inclusion of a treatment to attack the hypnozoite reservoir and will require mass administration regardless of the presence of blood-stage infections to target the undetectable parasite reservoir.

Currently, PQ is the only licensed drug with activity on the hypnozoite stage capable of preventing relapses [72]. However, due to its association with haemolysis in individuals with G6PD deficiency [72,73] and its long dosing schedule (up to 14 d), this drug is not in widespread use in many endemic areas, and WHO currently advises against PQ treatment without prior G6PD deficiency testing. In the absence of a reliable and affordable point-of-care G6PD test, the routine use of PQ for treatment thus remains a challenge in many endemic regions.

The challenges of implementing MDA with PQ are even larger, although not insurmountable [74]. A new generation of G6PD tests are being developed [75,76], and the arrival of tafenoquine, a long-acting 8-aminoquinoline that can be given as a single dose [34,76], will not only make MDA logistically simpler, but our modelling also predicts that MDA with tafenoquine will be more effective than regimens using PQ. Nevertheless, the development of alternative anti-hypnozoite treatments remains an important research priority for the elimination of *P. vivax* [16].

The treatment schedule used in the present study was optimised for the best possible anti-hypnozoite activity. The results from the clinical trial are therefore not directly indicative of the effectiveness of an MDA programme with standard 14-d PQ dosing. As a consequence, we assumed a significantly lower PQ efficacy (75%) in our model than we found in our study. Similarly, our results refer to the fast-relapsing *P. vivax* strains that are found in relatively highly endemic areas in the southwest Pacific, southeast Asia, and parts of Amazonia [77]. Further studies in areas with lower transmission levels and longer relapse intervals will be required to determine the generalisability of our finding of relapses causing 80% of infections and to extend the model predictions to other geographical regions and transmission settings.

In conclusion, this study demonstrates that relapsing infections are the overwhelming source (i.e., ~80%) of not only *P. vivax* blood-stage infections in children but also clinical episodes, and that they contribute substantially to maintaining transmission. Given the very ambitious timelines set by political leaders of *P. vivax*–endemic regions and limited funding, it is essential that scarce resources available for both *P. vivax* research and elimination be optimally allocated. The development of novel anti-hypnozoite drugs and interventions that can specifically target the hypnozoite reservoir are of highest priority. However, by predicting that MSAT programmes will not be effective in reducing the burden of *P. vivax* in affected populations, the models presented here also suggest that it is not worthwhile investigating or implementing MSAT programmes in *P. vivax*–endemic countries. Instead, efforts should now be directed towards the development of approaches for MDA programmes targeting areas and risk groups with confirmed local transmission.

Supporting Information

S1 Fig. Schematic representation of the *P. vivax* transmission model and the associated system of differential equations. S_0 denotes fully susceptible humans, I_0 denotes individuals with blood-stage infection, S_L denotes individuals with liver-stage infection with hypnozoites, and I_L denotes individuals with blood-stage infection and liver-stage infection with hypnozoites.
(TIF)

S2 Fig. The effect of two rounds of MDA and MSAT with anti-malarial drugs on *P. vivax* and *P. falciparum* blood-stage parasite prevalence, as predicted by the deterministic model. The grey and green shaded bars denote the duration of prophylactic protection for DHA-PIP/CQ and tafenoquine, respectively, after each treatment round. DHA-PIP and CQ were assumed to be administered as part of a 3-d regimen providing prophylaxis for 1 mo. PQ was assumed to be administered as part of a 14-d regimen providing prophylaxis for 15 d. Tafenoquine was assumed to be administered via a single dose providing prophylaxis for 2 mo.
(TIF)

S1 Table. Description of parameters for the malaria transmission model.
(PDF)

S1 Text. Trial protocol.
(PDF)

S2 Text. CONSORT checklist.
(PDF)

S3 Text. Overview of the malaria transmission model.
(PDF)

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Author Contributions

Conceived and designed the experiments: IM LS QB IF PS. Performed the experiments: LJR RW IB BK AW NH LL NT JB. Analyzed the data: CSNLWS SK MS LS MW LJR IM. Contributed to the writing of the manuscript: IF SK RW MS QB CSNLWS NH. All authors have read, and confirm that they meet, ICMJE criteria for authorship. Wrote the first draft of the manuscript: LJR SK MW IM. Agree with the manuscript's results and conclusions: LJR RW IB MW SK BK CSNLWS AW NH JB LL NT LS MS QB PS LS IF IM. Enrolled patients: BK IB LJR.

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Identification and Deconvolution of Cross-Resistance Signals from Antimalarial Compounds Using Multidrug-Resistant *Plasmodium falciparum* Strains

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***Plasmodium falciparum*, the most deadly agent of malaria, displays a wide variety of resistance mechanisms in the field. The ability of antimalarial compounds in development to overcome these must therefore be carefully evaluated to ensure uncompromised activity against real-life parasites. We report here on the selection and phenotypic as well as genotypic characterization of a panel of sensitive and multidrug-resistant *P. falciparum* strains that can be used to optimally identify and deconvolute the cross-resistance signals from an extended panel of investigational antimalarials. As a case study, the effectiveness of the selected panel of strains was demonstrated using the 1,2,4-oxadiazole series, a newly identified antimalarial series of compounds with *in vitro* activity against *P. falciparum* at nanomolar concentrations. This series of compounds was to be found inactive against several multidrug-resistant strains, and the deconvolution of this signal implicated *pfcrt*, the genetic determinant of chloroquine resistance. Targeted mode-of-action studies further suggested that this new chemical series might act as falcipain 2 inhibitors, substantiating the suggestion that these compounds have a site of action similar to that of chloroquine but a distinct mode of action. New antimalarials must overcome existing resistance and, ideally, prevent its *de novo* appearance. The panel of strains reported here, which includes recently collected as well as standard laboratory-adapted field isolates, is able to efficiently detect and precisely characterize cross-resistance and, as such, can contribute to the faster development of new, effective antimalarial drugs.**

Since the beginning of this century, the revived interest of the international community in the control of malaria, together with the 2007 call for an eradication agenda, resulted in a significant decrease in the burden of mortality and morbidity associated with this parasitic disease (2–4). Over the past 12 years, the global malaria mortality rate has decreased by an estimated 45%, averting 3.3 million deaths, 90% of which were among children under 5 years of age in sub-Saharan Africa (7). This has been achieved, at least in part, by the increased availability of safe and efficacious antimalarial chemotherapies. In 2012, 331 million courses of artemisinin-based combination therapies (ACTs), recommended by the WHO for first-line treatment of uncomplicated *Plasmodium falciparum* malaria, were delivered to the public and private sectors (7). However, in the absence of a fully protective antimalarial vaccine, the development and spread of drug resistance by *P. falciparum* are likely to constitute serious obstacles to the eradication of malaria.

The ability of *P. falciparum* to develop resistance mechanisms had a major impact on the efficacy of two former mainstay therapies for uncomplicated malaria, chloroquine and the sulfadoxine-pyrimethamine combination, whose use for the curative treatment of infections caused by this species had to be abandoned (11, 12). Before then, the prolonged use of partially ineffective chloroquine treatments in sub-Saharan Africa is reported to have caused an increase in malaria mortality of up to 3-fold (14).

ACTs are now threatened by the emergence of *P. falciparum* strains with reduced sensitivity to artesunate, a phenotype that was first reported in Southeast Asia in 2008 and that has spread locally since then (16–18). Failures of artesunate-mefloquine treatment and, more recently, dihydroartemisinin-piperaquine treatment have been reported in areas of artemisinin resistance (19–23). This suggests that the therapeutic efficacy of ACT might

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be compromised, which is a major concern, considering that no alternative treatments are ready to be deployed (25).

Resistance to essentially all antimalarial drugs previously or currently used to treat *P. falciparum* malaria have been identified to various degrees and levels of geographical spread (6). Moreover, resistance to new antimalarial compounds currently in clinical development can often be selected for *in vitro*, demonstrating the genetic ability of *P. falciparum* to acquire resistance to these compounds and suggesting that none would be intrinsically refractory to resistance if they happened to be deployed as new antimalarial therapies (27–29). Thus, measures need to be taken at all stages of the drug development process to minimize liabilities due to resistance and to maximize the life span over which antimalarial therapies have efficacy.

The Medicines for Malaria Venture (MMV) is a not-for-profit organization with the mission to discover, develop, and facilitate the delivery of new, effective, and affordable antimalarial drugs (31). Recently, a semiquantitative *in vitro* framework was developed to evaluate the resistance liabilities of new antimalarials and is currently used in MMV's collaborative projects to identify and deprioritize compounds for which the risk of the development of resistance is overt early in development (15). The underlying strategy is 2-fold: first, to evaluate if new antimalarial compounds show cross-resistance with previously or currently marketed antimalarials, as evidenced by a loss of *in vitro* activity due to mechanisms of resistance known to operate in natural parasite populations, and second, to evaluate the propensity of *de novo* mechanisms of resistance to new compounds to be selected *in vitro*. The first goal is achieved by measuring the *in vitro* activity of compounds against a panel of standard sensitive and multidrug-resistant (MDR) strains which were selected to represent strains with the major resistance mechanisms known to operate in the field (15). In this study, we deployed this selected panel of strains to determine if they accurately displayed the intended resistance phenotypes, effectively identified signs of cross-resistance to new antimalarial compounds, and allowed the deconvolution of the determinants of the cross-resistance signals. We present and discuss the use of this approach for the detailed evaluation of the cross-resistance of a new antimalarial chemical series identified by high-throughput compound screening.

MATERIALS AND METHODS

***Plasmodium falciparum* strain culture and drug assays.** *P. falciparum* strains NF54, D6, HB3, FCB, 7G8, K1, Dd2, and V1/S were obtained from the Malaria Research and Reference Reagent Resource Center (MR4; www.mr4.org). Strain TM90C2B was provided by Dennis E. Kyle (University of South Florida). These strains were maintained *in vitro* using the method of Trager and Jensen (35). Parasite growth after exposure to serial dilutions of antimalarial compounds was assessed using the [^{3}H]hypoxanthine incorporation assay, and the level of inhibition of growth is expressed as the median (50%) inhibitory concentration (IC_{50}) (36, 37). Exposures of 72 h were performed to allow the detection of slow-acting compounds and to enhance data reproducibility (38).

The multiclonal parasite lines HL1210 and HL1212 were isolated from patients presenting to the Hospital for Tropical Diseases in London, United Kingdom, in 2012 after returning from travel to Ghana and Nigeria, respectively, and are described in detail elsewhere (30). Both strains originated from areas of endemicity where ACT has been in use since the mid-2000s and thus likely have prior exposure to lumefantrine, dihydroartemisinin, and potentially other ACT partner drugs. These strains were maintained *in vitro*, and drug assays were performed as described in reference 30. These lines have been made publicly available at the European Malaria Reagent Repository (http://www.malariaresearch.eu/).

***Plasmodium falciparum* sequencing.** DNA for sequencing was obtained from 10 ml of nonsynchronized cultures at approximately 5% parasitemia. Cells were spun down, the pellet was lysed with 0.5% saponin, and the parasites were subsequently spun down and lysed in the lysis buffer contained in the Qiagen DNA purification kit. Nested PCRs were set up for the gene domains in question using the primers listed in Table S1 in the supplemental material and the conditions indicated in the Materials and Methods section in the supplemental material. PCR products were cleaned using a Qiagen PCR cleanup kit and sent to Macrogen (Amsterdam, The Netherlands) for bidirectional sequencing using the same primers used for the nested reaction. All sequences were aligned with the strain 3D7 reference sequences, and single nucleotide polymorphisms were called only if they were present on both strands. *pfmdr1* copy numbers were determined by real-time PCR as described previously (39).

Synthesis and characterization of compounds 1, 2, and 3. The synthesis and characterization of compounds 1, 2, and 3 of the 1,2,4-oxadiazole series are presented in the Materials and Methods section in the supplemental material. Compound 3 was synthesized in a limited amount and could not be included in all experiments reported here.

***Saccharomyces cerevisiae* heterologous system.** *DFR1*, the gene encoding dihydrofolate reductase (DHFR) from *Saccharomyces cerevisiae* (*ScDHFR*), was PCR amplified from genomic DNA templates and cloned into pCM188 to generate pCMScDHFR (5). The DHFR-coding sequence from *Plasmodium falciparum* (*PfDHFR*) with a codon usage suitable for expression in yeast was synthesized by Geneart and was subcloned into pCM188 (1) to generate pCMPfDHFR (5). The DHFR mutations N51I, C59R, and S108N, which confer resistance to antifolates in wild *Plasmodium falciparum* populations, were introduced by site-directed mutagenesis into pCMPfDHFR to generate pCMPfRdhfr$^{N51I,\ C59R}$, pCMPfRdhfr^{S108N}, and pCMPfRdhfr$^{N51I,\ C59R,\ S108N}$. pCM constructs were transformed into a BY4743-derived strain (*pdr5*::*HisMX*/*PDR5 dfr1*::*KanMX*/*DFR1 MATa*/*MATα his3Δ1*/*his3Δ1 leu2Δ0*/*leu2Δ0 met15Δ0*/*MET15 LYS2*/*lys2Δ0 ura3Δ0*/*ura3Δ0*), and following sporulation and tetrad dissections, spores with the genotype *pdr5*::*HIS3MX dfr1*::*KanMX MATα his3Δ1 leu2Δ0 MET15 lys2Δ0 ura3Δ0*/pCM were selected for use in drug sensitivity assays.

Standard yeast growth conditions and either YPD (2% peptone, 1% yeast extract, 2% glucose), supplemented sporulation medium (1% potassium acetate, 0.1% yeast extract, 0.05% glucose, amino acid supplements, 2% Bacto agar), or YNB-glucose (0.68% yeast nitrogen base, 2% ammonium sulfate, 2% glucose, 0.015% leucine) were used for all assays.

Serial dilutions (5 times) of stationary-phase cultures were prepared in 96-well plates and replicated onto YNB-glucose agar plates with or without drugs. Cells were allowed to grow for 24 h at 30°C and then photographed.

***In vitro* hemozoin formation and falcipain 2 enzyme activity assays.** Falcipain 2 activity in the presence of compounds 1 and 2 and chloroquine (5 μM) was assessed using a fluorometric assay, where the release of 7-amino-4-methyl coumarin (AMC) was monitored (excitation, 355 nm; emission, 460 nm) over 30 min at room temperature using an LS50B Perkin-Elmer fluorimeter in a buffer containing 100 mM sodium acetate, pH 5.5, 10 mM dithiothreitol, 7 μM fluorogenic substrate Z-Phe-Arg-AMC, and 200 nM enzyme. Falcipain 2 activity in the absence of any drug was taken as a positive control. Details on the enzyme expression and hemozoin formation assays are presented in the Materials and Methods section in the supplemental material.

***In vitro* abiotic β-hematin formation assay.** The β-hematin formation inhibition assay method described by Carter and colleagues was modified for manual liquid delivery (8, 9). Solutions of the samples in dimethyl sulfoxide were incubated in 96-well plates with NP-40 detergent (30.55 μM) and hematin (100 μM) in an acetate buffer (1 M, pH 4.8) for 5 to 6 h at 37°C. The detection of free heme was based on the pyridine-

TABLE 1 Amino acid sequences of selected gene polymorphisms involved in *P. falciparum* drug resistance

	Amino acid at the indicated position in the following gene[a]:																			
	pfcrt					*pfmdr1*[b]					*pfdhfr*					*pfdhps*				*pfcytb*,
Strain	72	73	74	75	76	86	184	1034	1042	1246	16	51	59	108	164	436	437	581	613	268
Wild type	C	V	M	N	K	N	Y	S	N	D	A	N	C	S	I	S	A	A	A	Y
NF54	C	V	M	N	K	N	Y	S	N	D	A	N	C	S	I	F	G	A	A	Y
D6	C	V	M	N	K	N	Y	S	N	D	A	N	C	S	I	A	A	A	A	Y
HB3	C	V	M	N	K	N	F	S	D	D	A	N	C	N	I	F	A	A	A	Y
FCB	C	V	I	E	T	Y	Y	S	N	D	T	N	C	T	I	F	A	A	A	Y
7G8	S	V	M	N	T	N	F	R	D	Y	A	I	C	N	I	F	G	A	A	Y
K1	C	V	I	E	T	Y	Y	S	N	D	A	N	R	N	I	S	G	G	A	Y
Dd2	C	V	I	E	T	F	Y	S	N	D	A	I	R	N	I	F	G	A	S	Y
V1/S	C	V	I	E	T	Y	Y	S	N	D	A	I	R	N	L	F	G	A	T	Y
TM90C2B	C	V	I	E	T	N	F	S	N	D	A	I	R	N	L	F	G	G	A	S
HL1210[c]	C	V	I	E	T	Y	F	S	N	D	A	I	R	N	I	S	G	A	A	n/a
HL1212[c]	C	V	M	N	K	N	F	S	N	D	A	I	R	N	I	S	G	A	A	n/a

[a] Mutant residues are shaded.
[b] The *pfmdr1* copy number was 1 for the wild-type strain and strains NF54, D6, HB3, 7G8, K1, V1/S, TM90C2B, HL1210, and HL1212; 4 for FCB; and 3 for Dd2.
[c] As reported in reference 30.

ferrichrome method developed by Ncokazi and Egan (10). The detailed assay procedure has been reported previously (13).

RESULTS

Genotypic validation of selected *P. falciparum* strains. The selected panel of *P. falciparum* strains included the MDR strains HB3, FCB, 7G8, K1, Dd2, V1/S, and TM90C2B as well as the sensitive strains NF54 and D6, all of which were selected on the basis of genotypic and phenotypic information previously reported in the literature (15).

In order to evaluate the ability of these strains to identify cross-resistance signals, genes involved in drug resistance were sequenced to confirm the presence of specific mutations known to cause resistance to various antimalarial compounds (Table 1). These genes were those for the *P. falciparum* chloroquine resistance transporter (*pfcrt*), multidrug resistance 1 (*pfmdr1*), dihydrofolate reductase (*pfdhfr*), dihydropteroate synthase (*pfdhps*), and cytochrome *b* (*pfcytb*). Mutations of *pfcrt* and *pfmdr1* have been shown to mediate resistance to chloroquine and amodiaquine, mutations of *pfdhfr* have been shown to mediate resistance to pyrimethamine and cycloguanil, mutations of *pfdhps* have been shown to mediate resistance to sulfadoxine, and mutations of *pfcytb* have been shown to mediate resistance to atovaquone. The expected *pfcrt* and *pfcytb* genotypes were observed, and minor differences from the mutations reported in the literature were observed for *pfmdr1*, *pfdhfr*, and *pfdhps* (see the references in reference 15). Regarding *pfmdr1*, strain HB3 was found to carry the mutation Y184F, while this residue was expected to be wild type; strain Dd2 had the mutation N86F instead of N86Y, and strain 7G8 had the mutation S1034R instead of S1034C. Four copies of the *pfmdr1* gene (instead of two) and three copies of the *pfmdr1* gene (instead of four) were identified in strains FCB and Dd2, respectively. FCB was also found to express the *pfdhfr* mutation A16T instead of A16V. Finally, strains NF54, HB3, and 7G8 were found to carry the *pfdhps* mutation S436F instead of the wild-type residue.

Phenotypic validation of selected *P. falciparum* strains. The presence of the expected resistance phenotypes in the panel of strains was determined by measuring their sensitivity to various standard antimalarial compounds during an *in vitro* exposure of 72 h. Artesunate, atovaquone, chloroquine, cycloguanil, mefloquine, and pyrimethamine were evaluated, and resistance was considered to occur if the median (50%) inhibitory concentration (IC_{50}) of a given strain was found to be, on average, more than 10-fold greater than that of the sensitive reference strain NF54 (Fig. 1; see Table S2 in the supplemental material). This threshold, which is only of an indicative nature, was selected to be in the range of the smallest IC_{50} shifts associated with clinical resistance (e.g., for chloroquine [40]), while it simultaneously avoided a false-positive resistance signal due to the intrinsic variation of phenotypic assays.

None of the strains tested showed resistance, as defined here, to artesunate and mefloquine. As expected, TM90C2B was the only strain resistant to atovaquone. NF54, D6, HB3, and 7G8 were found to be sensitive to chloroquine; however, the phenotype of 7G8 was close to the resistance threshold, with 7G8 having an 8.3-fold greater tolerance to the drug than N54. FCB, K1, Dd2, V1/S, and TM90C2B all displayed resistance to chloroquine. All strains except NF54, D6, and HB3 were found to be resistant to cycloguanil, while resistance to pyrimethamine was apparent in all strains except NF54, D6, and FCB. The selected strains effectively displayed various resistance phenotypes and therefore represent an appropriate tool for evaluating the potential cross-resistance of antimalarial compounds with a variety of existing molecular mechanisms of resistance.

Case study with the 1,2,4-oxadiazole series. To further evaluate the ability of the selected panel of strains to identify and facilitate the characterization of cross-resistance signals from new antimalarial compounds, we studied three analogue compounds (compounds 1, 2, and 3). These three molecules comprise a 1,2,4-oxadiazole moiety, a 1,4-diazepam amide, a tertiary piperidine, and an amide group, and we commonly refer to them as the "1,2,4-oxadiazole" series (Fig. 2A). Compounds 1 and 2 were initially identified as hits on the erythrocytic stage of *P. falciparum* during the phenotypic screening of a library of more than 250,000 compounds (24). Compound 3 was synthesized while performing the resynthesis of compounds 1 and 2. The IC_{50}s for these three

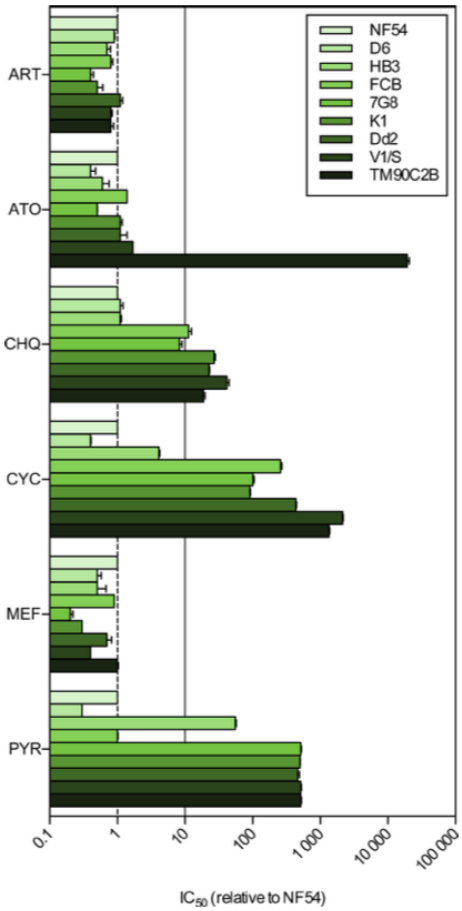

FIG 1 Phenotypic validation of the panel of MDR strains. The relative IC_{50}s of artesunate (ART), atovaquone (ATO), chloroquine (CHQ), cycloguanil (CYC), mefloquine (MEF), and pyrimethamine (PYR) against the panel of *P. falciparum* laboratory strains are shown. The solid line shows a 10-fold increase in the IC_{50} relative to that for NF54 ($n \geq 2$; error bars are SDs).

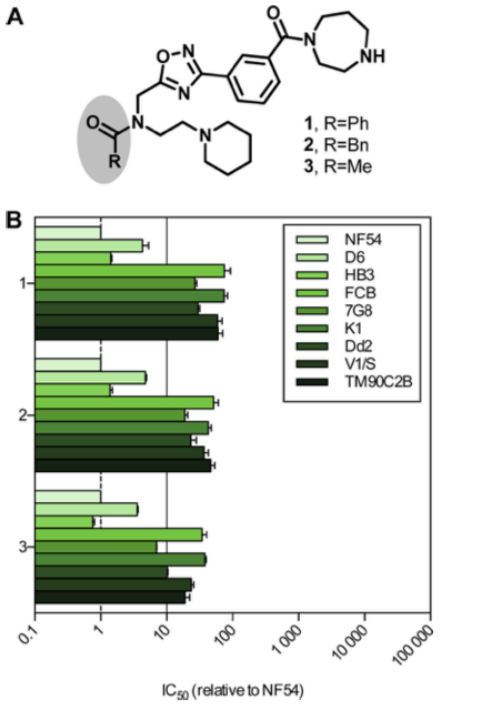

FIG 2 Resistance profile of the 1,2,4-oxadiazole series. (A) Structures of the three compounds from the 1,2,4-oxadiazol series. Ph, phenyl; Bn, benzyl; Me, methyl. (B) Relative IC_{50}s of compounds 1, 2, and 3 against a panel of *P. falciparum* laboratory strains. The solid line shows a 10-fold increase in the IC_{50} relative to that for NF54 ($n \geq 2$; error bars are SDs).

compounds tested against the sensitive strain NF54 ranged from 60 nM to 278 nM (Table 2). This, together with favorable physicochemical properties, suggested that this novel chemical scaffold might be a valuable starting point for a hit-to-lead medicinal chemistry program. As part of a standard resistance risk assessment and as a first filter, the activity of these compounds against multidrug-resistant strain K1 was measured. A strong resistance signal was identified, with the K1 IC_{50}s ranging from 3.8 μM to 7.5 μM, representing, for each compound, an increase in the IC_{50} of more than 20-fold compared to the values for NF54 (Table 2). The activity of these compounds was further profiled using the complete panel of *P. falciparum* strains with the objective of identifying the mechanism of resistance responsible for this loss of potency against K1.

The relative activity of compounds 1, 2, and 3 against the panel of strains is reported in Fig. 2B (absolute values are given in Table S3 in the supplemental material). All three compounds were found to be active against D6 and HB3. Compounds 1 and 2 were inactive against all the other strains tested, that is, FCB, 7G8, K1, Dd2, V1/S, and TM90C2B. Compound 3 displayed a similar lack of activity against these strains, with the exception of 7G8, for which the IC_{50} increased by only 7-fold. The overall profiles of resistance to the three compounds were very similar. First, the D6 IC_{50} was slightly elevated; second, a further decrease in activity against 7G8 and Dd2, resulting in resistance, as defined here, except for the activity against 7G8 in the case of compound 3 was detected; and finally, a complete loss of activity against the remaining strains (FCB, K1, V1/S, and TM90C2B) was found.

Compared to the resistance profiles obtained with standard

TABLE 2 Activity of the 1,2,4-oxadiazole series compounds against the NF54 and K1 *P. falciparum* strains

Compound	IC_{50} (nM)[a]		Ratio of IC_{50} for K1/IC_{50} for NF54
	NF54	K1	
Chloroquine	10	157	16
1	60	4,972	83
2	163	3,813	23
3	278	7,465	27

[a] Values are from one representative experiment.

antimalarials, the 1,2,4-oxadiazole series appeared to be cross-resistant with two compounds, chloroquine and cycloguanil, suggesting that genetic determinants of resistance to this series might be mutations in *pfcrt* and *pfdhfr*. All the strains fully sensitive to this series of compounds carry a wild-type *pfcrt* allele, whereas the CVIET or SVMNT mutants displayed an elevated IC_{50} that was beyond our threshold of resistance in the vast majority of cases. Similarly, all resistant strains contained at least two mutations at *pfdhfr* codon 16, 51, 59, 108, or 164, while the sensitive ones were either wild type (NF54, D6) or contained only a single S108N mutation (HB3). Conversely, no correlation between specific mutations of *pfmdr1*, *pfdhps*, and *pfcytb* or copy number variations of *pfmdr1* and the sensitivity of specific strains to members of the 1,2,4-oxadiazole series was apparent.

In order to further discriminate between the *pfcrt* and *pfdhfr* mutations as potential determinants for resistance to compounds 1, 2, and 3, their activity was evaluated in a heterologous system of *Saccharomyces cerevisiae* strains expressing wild-type or mutated versions of the *P. falciparum dhfr* gene (1). The growth of yeast strains expressing *S. cerevisiae dhfr* (*ScDHFR*), the wild-type *P. falciparum dhfr* (*PfDHFR*), or the mutant alleles $Pfdhfr^{N51I,\ C59R}$, $Pfdhfr^{S108N}$, and $Pfdhfr^{N51I,\ C59R,\ S108N}$ was determined in the presence of compounds 1, 2, and 3 and cycloguanil (see Fig. S1 in the supplemental material). Cycloguanil specifically inhibited the growth of yeast strains expressing the wild-type PfDHFR enzyme, and this effect was partially lost with the $Pfdhfr^{N51I,\ C59R}$ and $Pfdhfr^{S108N}$ alleles and totally lost with the triple mutant, $Pfdhfr^{N51I,\ C59R,\ S108N}$. On the other hand, compounds 1, 2, and 3 failed to inhibit the growth of the yeast strain expressing *PfDHFR*, and no difference between strains expressing wild-type or mutated versions of *PfDHFR* was noticeable. These data suggest the absence of cross-resistance between cycloguanil and the 1,2,4-oxadiazole series and appear to rule out *dhfr* mutations as the genetic determinants of resistance to members of this chemical series observed.

We next reasoned that if resistance to the 1,2,4-oxadiazole series was mediated by *pfcrt* mutations, it might be reversed by verapamil, similar to the findings for chloroquine resistance (26). We exposed the *pfcrt* mutant and chloroquine-resistant strain K1 to either 0, 50, or 100 ng/ml of verapamil and determined the IC_{50} of artesunate (negative control), chloroquine (positive control), and compounds 1, 2, and 3 under these conditions (see Fig. S2 in the supplemental material). As expected, the sensitivity of K1 to chloroquine, but not to artesunate, increased in a dose-dependent manner with concomitant incubation with verapamil. The IC_{50}s of compounds 1 and 2 did not change in response to verapamil. The sensitivity of strain K1 to compound 3 increased in response to incubation with verapamil at concentrations of 50 ng/ml and 100 ng/ml, but to an extent lower than that for chloroquine (the IC_{50}s obtained by incubation with 100 ng/ml verapamil decreased by 29% and 51% compared to those with no verapamil for compound 3 and chloroquine, respectively).

The fact that verapamil did not significantly sensitize K1 to the 1,2,4-oxadiazole series suggests that *pfcrt* might not be the prime genetic determinant of resistance to this series. An alternative hypothesis is that the level of resistance to these compounds is too high to be reversed by verapamil. The IC_{50}s of compounds 1, 2, and 3 for strain K1 ranged from 3.0 μM to 11.0 μM, whereas the IC_{50} of chloroquine for K1 was 1 order of magnitude lower, at 0.12 μM. The relative shift in the IC_{50} compared to the NF54 IC_{50} was also less for chloroquine, at 16-fold, whereas it was 74-, 43-, and 38-fold for compounds 1, 2, and 3, respectively. Alternatively, higher verapamil concentrations might be required to induce a reversion of the resistance of the *pfcrt* mutant parasites to the 1,2,4-oxadiazole series.

TABLE 3 Sensitivity of recently laboratory-adapted *P. falciparum* isolates to the 1,2,4-oxadiazole series compounds

Compound	IC_{50} (nM)[a]		Ratio of IC_{50} for HL1210/IC_{50} for HL1212
	HL1210	HL1212	
Chloroquine	150.0 ± 7.0	12.0 ± 3.0	12.5
Artesunate	4.2 ± 1.1	7.1 ± 1.2	0.6
Pyrimethamine	>10,000.0	>10,000.0	NA[b]
Cycloguanil	879.0 ± 218.0	1,877.0 ± 156.0	0.5
1	2,270.0 ± 548.0	33.0 ± 10.0	68.8
2	2,694 ± 502.0	44.0 ± 11.0	61.3
3	>10,000.0	98.0 ± 26.0	>100

[a] Data are means and standard errors of the means from at least five independent experiments.
[b] NA, not applicable.

To evaluate the implication of *pfcrt* mutations in the resistance to this chemical series in a more direct manner, we took advantage of *P. falciparum* field isolates recently adapted to laboratory culture and fully characterized for key determinants of resistance (30). More particularly, we selected isolates HL1210 and HL1212, which share the same *pfdhfr* triple mutation haplotype and differ in their *pfcrt* sequences. HL1210 is a *pfcrt* mutant (CVIET), and HL1212 is a *pfcrt* wild-type strain (CVMNK) (Table 1). The chloroquine IC_{50} observed for these two strains confirmed the resistance and sensitivity status of HL1210 and HL1212, respectively (Table 3). Both strains were equally susceptible to artesunate and resistant to pyrimethamine and cycloguanil, as expected. The activity of the 1,2,4-oxadiazole series mirrored that of chloroquine rather than that of the DHFR inhibitors pyrimethamine and cycloguanil, with a 60-fold or greater increase in activity against *pfcrt* wild-type strain HL1212 than *pfcrt* mutant HL1210 (Table 3). These results suggest that mutated PfCRT might play a direct role in *P. falciparum* resistance to the 1,2,4-oxadiazole series and, by extension, that the mode of action of these compounds could reside in the digestive vacuole, from which mutant forms of the PfCRT transmembrane transporter are postulated to exclude chloroquine in resistant parasites (32, 33).

The mode of action of chloroquine has been linked with the inhibition of proteases involved in the degradation of hemoglobin as well as with the prevention of hemozoin formation, possibly via heme binding, in the digestive vacuole (see Fig. S3A in the supplemental material) (34). In order to evaluate if compounds from the 1,2,4-oxadiazole series might also interfere with the hemoglobin degradation pathway, we measured the activity of compounds 1 and 2 in an *in vitro* hemozoin formation assay based on enzymatic or autocatalytic reactions (see Fig. S3B to D and Table S4 in the supplemental material). *In vitro*, heme can be released from hemoglobin by falcipain 2 and subsequently converted to hemozoin by the heme detoxification protein (HDP) or via an autocatalytic mechanism (34, 41). The autocatalytic formation of hemozoin appears to be slightly inhibited by compounds 1 and 2 (IC_{50}s, 452 μM and 587 μM, respectively, compared to an IC_{50} of 43.5 μM for amodiaquine; see Table S4 in the supplemental material). Prein-

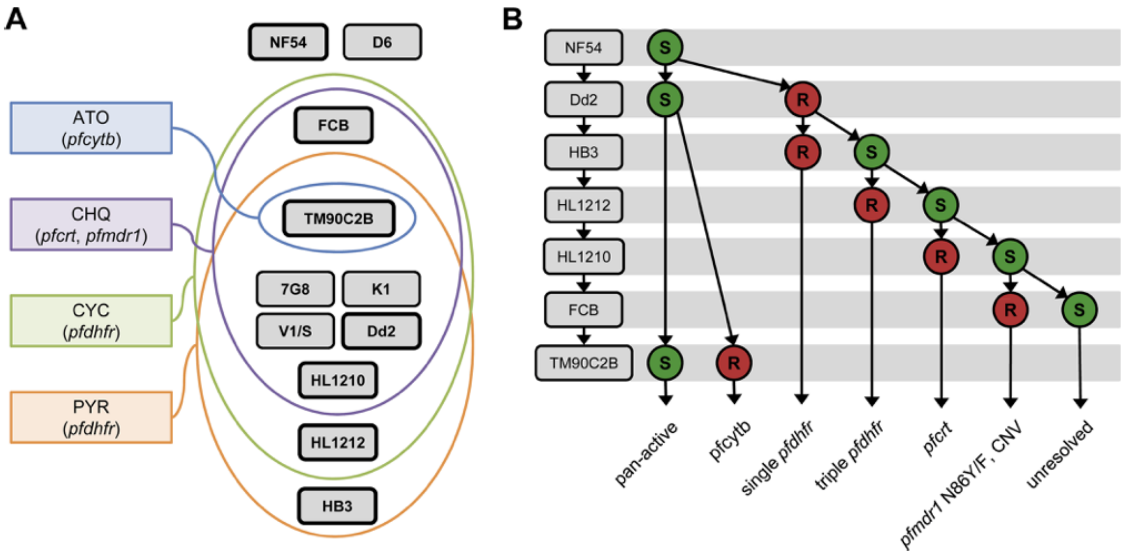

FIG 3 Cross-resistance deconvolution. (A) Venn diagram of the profiles of resistance of the *P. falciparum* strains to atovaquone (ATO), chloroquine (CHQ), cycloguanil (CYC), and pyrimethamine (PYR). The main genetic determinants of resistance for each compound is indicated, and the strains in boxes with bold borders correspond to the ones evaluated in the assay whose results are presented in panel B. (B) Deconvolution cascade of cross-resistance signals determined using the multidrug-resistant *P. falciparum* strain panel. R, resistant; S, sensitive; CNV, copy number variation.

cubation of HDP with compounds 1 and 2 did not inhibit the formation of hemozoin (see Fig. S3B in the supplemental material). However, the activity of falcipain 2 appeared to be markedly inhibited by compounds 1 and 2 (5 μM) in a fluorometric catalytic assay; in contrast, chloroquine did not have any effect on falcipain 2 activity *per se* (see Fig. S3C in the supplemental material). In addition, to assess the effect of compounds 1 and 2 on the released heme, they were added to a hemoglobin-to-hemozoin conversion assay. The addition of either compound did not alter the percentage of hemozoin formation, in contrast to chloroquine, which bound free heme and blocked hemozoin formation (see Fig. S3D in the supplemental material). Altogether, these observations suggest that compounds of the 1,2,4-oxadiazole series might be competitive inhibitors of the cysteine protease falcipain 2, a mode of action distinct from that of chloroquine but similarly located in the food vacuole, and thus, the findings are in line with a potential mode of resistance mediated by *pfcrt*.

A test cascade for resistance deconvolution. In order to obtain the maximum amount of information about potential *in vitro* cross-resistance mechanisms, a given set of strains should ideally provide the highest level of resistance specificity. In other words, it is desirable to occupy the maximum possible number of intersections when representing resistance to standard antimalarial compounds as spaces in a Venn diagram (Fig. 3A). Such a graphical representation illustrates that the combination of identical *pfdhfr* sequences and divergent *pfcrt* sequences was absent from the selected panel of standard *P. falciparum* laboratory strains and that this combination is needed to resolve cases of resistance implicating strains with mutations in both these genes, as is the case with compounds 1, 2, and 3. This limitation was circumvented by the addition of HL1210 and HL1212 to the panel.

On the basis of the genotypic and phenotypic data reported here, it is possible to devise an optimal deconvolution scheme that allows acquisition of the maximum amount of information by testing a minimal number of strains (Fig. 3B). At least one strain from each intersection represented in Fig. 3A was included in this scheme. Applying this signal deconvolution to the 1,2,4-oxadiazole series readily identified *pfcrt* to be a potential genetic determinant of the resistance associated with this series. More generally, compounds active against Dd2 as well as TM90C2B are likely to be pan-active, whereas a resistance signal for Dd2 should trigger the sequential testing of additional strains in order to provide information about the potential genetic determinants of resistance.

DISCUSSION

The genotypic and phenotypic evaluation of a panel of sensitive and MDR *P. falciparum* strains previously selected to evaluate the cross-resistance potential of antimalarial compounds is reported here. Minor genotypic differences from those reported in the literature were identified for some residues of *pfmdr1*, *pfdhfr*, and *pfdhps* and also for the *pfmdr1* copy number. It is unknown whether the sequences reported here are the original haplotypes of these strains or if mutations were acquired during culture passages. Regardless, these are unlikely to significantly alter the respective resistance phenotypes of these strains.

Indeed, the phenotype-based resistance profiles correlated well with expectations on the basis of the findings in the literature as well as with genotypic data. All strains carrying a mutant *pfcrt* allele at codons 72 to 76 displayed *in vitro* resistance to chloroquine, according to our definition, with the exception of strain 7G8, the IC_{50} for which shifted by only 8.3-fold, as expected due to the South American-type (SVMNT) mutant allele of this strain, whereas the other mutant strains carries the Southeast Asian-type

allele (CVIET) (42). The SVMNT haplotype is best described as an amodiaquine-resistant genotype, known to impart some survival advantage after chloroquine treatment *in vivo*, but it does not lead to IC_{50} estimates as high as those due to the CVIET form of *pfcrt* in gene replacement studies (32). Strain TM90C2B was resistant to atovaquone and was the only strain with a Y268S mutation in PfCYTB. The responses to the DHFR inhibitors pyrimethamine and cycloguanil were also in line with the observed *pfdhfr* sequences. The strains that had wild-type sequences at codons 16, 51, 59, 108, and 164 (ANCSI), strains NF54 and D6, were sensitive to both compounds. Strains with the N51I, C59R, and S108N triple mutations, that is, strains K1, Dd2, V1/S, and TM90C2B, showed resistance to both compounds. The mutant with the single S108N mutation, HB3, showed resistance to pyrimethamine and not to cycloguanil, while the converse was true for the mutant with the single S108T mutation, FCB. The *pfdhps*-mediated resistance to sulfadoxine was not evaluated here because of the specific culture conditions required (43); however, it is expected, on the basis of the *pfdhps* sequence diversity in specific resistance-mediating residues, that cross-resistance with sulfonamides would be readily and specifically identifiable by using the panel of strains under appropriate culture conditions.

In this study, we defined resistance to be a greater than 10-fold shift in the IC_{50} compared to the IC_{50} for strain NF54. This threshold was arbitrary but small enough to be in line with the smallest IC_{50} shifts documented to be associated with clinical resistance and simultaneously large enough to avoid false-positive signals due to assay variations. An IC_{50} shift of that magnitude should be a cause for concern, warranting further investigations, such as the ones described here for the 1,2,4-oxadiazole series. The attempt to define more precisely drug-specific *in vitro* IC_{50} thresholds directly applicable to clinical situations is a challenging task for reasons described elsewhere (44). We favor a simpler and more conservative approach that is more relevant to antimalarial compounds in development for which no clinical data exist.

We have reported on a new antimalarial chemical series, the 1,2,4-oxadiazole series, to which MDR strain K1 was overtly resistant. Importantly, no analogues of these compounds could be found in the literature, and no hypothesis on their mode of action could be made. The complete profiling of three analogues with the panel of strains suggested that the resistance of *P. falciparum* to these compounds is likely to be mediated by *pfcrt* or *pfdhfr*. Further independent experimental approaches seem to favor a role of *pfcrt* over a role of *pfdhfr* but suggest that the mode of action of these compounds is different from that of chloroquine. In particular, the addition of *P. falciparum* isolates recently adapted for laboratory culture to the panel was necessary to provide further segregation between resistance mediated by *pfcrt* and that mediated by *pfdhfr*, revealing a shortcoming in the ability of the original panel of strains to fully deconvolute cross-resistance signals. These two isolates are also evolutionarily closer to the current target parasite populations than the standard laboratory strains, most of which were culture adapted more than 20 years ago (30).

Importantly, the panel of strains was not devised to include all known alleles of each resistance-mediating gene reported here, as it would require a very large number of strains to be tested, rendering screening activities largely ineffective. More than 20 different mutant *pfcrt* alleles, for instance, have been described to date (32). We favored the selection of a relatively limited number of strains to optimally represent the main causal mutations together with the largest possible diversity in order to facilitate the deconvolution of resistance signals with a manageable number of parasite strains. A direct corollary is that additional specific strains should be screened when a specific mode of resistance might be suspected on the basis of the structure of a compound or a specific activity pattern.

A limitation of our approach is that cross-resistance with artemisinins is not readily detectable with the current panel of *P. falciparum* strains, first, because none of them has been reported to be resistant to artemisinins and, second, because the standard *in vitro* IC_{50} determination assay using 72 h of drug exposure is not suited to the detection of this specific resistance phenotype (45). Consequently, it will be essential to include in the panel one or more of the artemisinin-resistant strains recently made available at MR4 (strains MRA-1236 to MRA-1241) and to profile the activities of new compounds against them using the recently developed ring-stage survival assay, which is able to replicate artemisinin resistance in the laboratory (45, 46). Along the same line, it would be important to determine the genotype of the strains discussed here for K13, a recently described genetic marker of artemisinin resistance (47). These strains were isolated either before the introduction of artemisinins or from areas where resistance has not yet been reported, and consequently, all are expected to be the K13 wild type, but this awaits confirmation. Characterization of the panel could also be reinforced by typing additional minor genetic polymorphisms involved in resistance mechanisms as compensatory mutations or secondary determinants, such as *gch1* copy number, *pfcrt* mutations outside codons 72 to 76, or *pfmrp1* polymorphisms (32, 48, 49).

More importantly and in order to stay as representative and effective as possible, the composition of an optimal panel will necessarily need to evolve with time to include strains with new resistance mechanisms emerging in the field. The recent report of apparent clinical resistance to piperaquine might prefigure such a case (23). Once they are available, field-isolated and validated piperaquine-resistant strains should be included in a revised test cascade.

Overall, the multiple sensitive and MDR *P. falciparum* strains characterized here can be used as a tool to identify cross-resistance with known naturally occurring resistance mechanisms rapidly and effectively. The panel can thus be used to facilitate the identification of pan-active compounds and to determine the genetic determinants of resistance to compounds displaying cross-resistance signals. By testing between 2 strains (for pan-active compounds) and 5 strains (for compounds displaying a *pfmdr1*-mediated or unresolved resistance), in addition to NF54, the test cascade presented here allows such information to be rapidly gained and facilitates early decision making during drug development (15, 50). An understanding of the mode of resistance to new antimalarial compounds can also potentially inform the design of new analogues to overcome it.

The mechanism of resistance against compounds of the 1,2,4-oxadiazole series, presented here, could successfully be related to *pfcrt*. This, in turn, guided the identification of the potential mode of action for these compounds to be through the inhibition of falcipain 2 in the food vacuole. Interestingly, the structure of the series does not contain the typical pharmacophore of cysteine protease inhibitors, and the apparent disconnect between falcipain 2 inhibition and whole-cell activity may be explained by the presence of two basic amines that would allow the compounds to be

concentrated in the acidic food vacuole by a pH gradient (51). Further studies might investigate the morphological changes to the parasite induced by compounds of the 1,2,4-oxadiazole series, as falcipain inhibitors have previously been associated with impaired hemoglobin digestion, which was visible by light microscopy (52). Analogues potentially able to overcome the *pfcrt*-mediated resistance might also be synthesized and tested.

Altogether, evaluation of the 1,2,4-oxadiazole case study series illustrates the breadth of information that can be gained by profiling new antimalarial compounds with this set of *P. falciparum* strains. Ultimately, the careful selection of compounds refractory to already existing resistance mechanisms is an essential step in the development of new antimalarial drugs, which is facilitated by the testing strategy developed here.

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EDUCATION

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Associated with University of Basel, Basel

Charité Berlin, Berlin: M.Sc. Molecular Medicine (1.0 / Excellent) 2009 - 2011

Technical University of Munich, Munich: B.Sc. Biochemistry (1.6 / With merit) 2006 - 2009

RESEARCH EXPERIENCE

Swiss Tropical and Public Health Institute, Basel June 2012 – present
Dpt. Medical Parasitology and Infection Biology, Molecular Diagnostics Unit
PhD candidate: "Infection dynamics of *P. falciparum* in children from Papua New Guinea."

Institute of Medical Research, Madang/Maprik, Papua New Guinea Aug 2015 – Oct 2015
Field work in context of PhD studies Sept 2013 – Feb 2014

HU Institute of Biology, Molecular Parasitology, Berlin Feb 2011 – Aug 2011
M.Sc. Thesis: "Testing the functional importance of DLC8a for the obligate intracellular parasite *Toxoplasma gondii*."

Institute of Cell Biology and Neurobiology, Berlin Sept 2010 – Dec 2010
Institute for Medical Genetics, Berlin April 2010 – July 2010
Institute of Experimental Endocrinology, Berlin Oct 2009 – Feb 2010
Term papers: "The role of CDK5RAP2 in primary autosomal recessive microcephaly."
"Functional analysis of *PTHLH* promoter mutations."
"Effects of 3-T1AM and T3 on thyroid hormone biosynthesis."

Institute of Virology, Viral Vector Vaccines Group, Munich April 2009 – July 2009
B.Sc. Thesis: "Generation and characterization of recombinant MVA vectors producing the fluorescent protein mCherry."

Max-Planck-Institute of Neurobiology, Molecular Neurology, Munich Feb 2008 – April 2008
Institute for Pharmacy and Molecular Biotechnology, Heidelberg Sept 2005 – Jan 2006

ADDITIONAL EXPERIENCE

PhD student representative June 2013 – Jan 2015
International PhD program in Infection Biology, Swiss Tropical and Public Health Institute

Student representative and assistant to program coordinators 2009 - 2011
M.Sc. Molecular Medicine program, Charité Berlin

SCIENTIFIC PUBLICATIONS

Robinson LJ, Wampfler R, Betuela I, Karl S, White MT, Li Wai Suen CSN, Hofmann NE, Kinboro B, Waltmann A, Brewster J, Lorry L, Tarongka N, Samol L, Silkey M, Bassat Q, Siba PM, Schofield L, Felger I, Mueller I. *Strategies for understanding and reducing the Plasmodium vivax and Plasmodium ovale hypnozoite reservoir in Papua New Guinean children: A randomised placebo-controlled trial and mathematical model.* PLoS Med. 2015 Oct 27;12(10)

Hofmann N, Mwingira F, Shekalaghe S, Robinson LJ, Mueller I, Felger I. *Ultra-sensitive detection of Plasmodium falciparum by amplification of multi-copy subtelomeric targets.* PLoS Med. 2015 Mar 3;12(3):e1001788.

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Qureshi BM & Hofmann N, Arroyo-Olarte RD, Nickl B, Hoehne W, Jungblut PR, Lucius R, Scheerer P, Gupta N. *Dynein light chain 8a of Toxoplasma gondii, a unique conoid-localized β-strand-swapped homodimer, is required for an efficient parasite growth.* FASEB J. 2013 Mar;27(3):1034-47

CONFERENCES

64th ASTMH Meeting, Philadelphia, USA **Oct 2015**
Oral presentation: "Infection dynamics of natural *Plasmodium* infections in PNG children."

11th Annual BioMalPar/EVIMalaR Conference, Heidelberg, Germany **May 2015**
Poster: "Molecular monitoring of malaria in PNG children after up-scaling of control efforts."

63rd ASTMH Meeting, New Orleans, USA **Oct 2014**
Poster: " Ultra-sensitive detection of *Plasmodium falciparum* by amplification of multi-copy subtelomeric targets reveals underestimation of parasite prevalence."

10th Annual BioMalPar/EVIMalaR Conference, Heidelberg, Germany **May 2014**
Poster: "Ultrasensitive detection of *Plasmodium falciparum* by amplification of multi-copy subtelomeric targets using quantitative PCR."

9th Annual BioMalPar/EVIMalaR Conference, Heidelberg, Germany **May 2013**
Oral presentation: "Molecular tools for monitoring transmission dynamics of *Plasmodium falciparum*"

PhD student meeting of the Swiss Society of Tropical Medicine and Parasitology (SSTMP), Bern, Switzerland **Dec 2012**
Oral presentation: "Infection and transmission dynamics of *Plasmodium* in children from PNG."

SKILLS

Languages
German: native language
English: fluent
Spanish: basic knowledge
Tok Pisin: basic knowledge

Software
Working knowledge in Microsoft Office
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Working knowledge in R

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