Force of infection is key to understanding the epidemiology of *Plasmodium falciparum* malaria in Papua New Guinean children

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Genotyping *Plasmodium falciparum* parasites in longitudinal studies provides a robust approach to estimating force of infection (FOI) in the presence of superinfections. The molecular parameter $_{mol}$FOI, defined as the number of new *P. falciparum* clones acquired over time, describes basic malaria epidemiology and is suitable for measuring outcomes of interventions. This study was designed to test whether $_{mol}$FOI influenced the risk of clinical malaria episodes and how far $_{mol}$FOI reflected environmental determinants of transmission, such as seasonality and small-scale geographical variation or effects of insecticide-treated nets (ITNs). Two hundred sixty-four children 1–3 y of age from Papua New Guinea were followed over 16 mo. Individual parasite clones were tracked longitudinally by genotyping. On average, children acquired 5.9 (SD 9.6) new *P. falciparum* infections per child per y. $_{mol}$FOI showed a pronounced seasonality, was strongly reduced in children using ITNs (incidence rate ratio, 0.49; 95% confidence interval, [0.38, 0.61]), increased with age, and significantly varied within villages (P = 0.001). The acquisition of new parasite clones was the major factor determining the risk of clinical illness (incidence rate ratio, 2.12; 95% confidence interval, [1.93, 2.31]). Adjusting for individual differences in $_{mol}$FOI completely explained spatial variation, age trends, and the effect of ITN use. This study highlights the suitability of $_{mol}$FOI as a measure of individual exposure and its central role in malaria epidemiology. It has substantial advantages over entomological measures in studies of transmission patterns, and could be used in analyses of host variation in susceptibility, in field efficacy trials of novel interventions or vaccines, and for evaluating intervention effects.

infection dynamics | cohort studies | molecular monitoring

Understanding the relationships between transmission intensity, prevalence of infection, and the incidence of clinical malaria is at the heart of malaria epidemiology. Transmission intensity is traditionally quantified through entomological measures of exposure, in particular the entomological inoculation rate (EIR) (1). However, accurately measuring EIRs is notoriously difficult and labor-intensive (2). Furthermore, prevalence (3, 4) and clinical incidence (5) depend on susceptibility as well as exposure to infectious stages of the parasite, and so these quantities may be more closely related to measures of transmission intensity determined by parasitological parameters within the human host.

The force of infection (FOI), that is, the number of *Plasmodium* infections acquired over time, has long been proposed as an alternative measure of transmission, and different approaches to measuring FOI have been proposed. For example, infant conversion rates (6) (i.e., time to a patent infection in uninfected children less than 1 y of age) were widely used in the 1950s–1970s. Although being an excellent marker for transmission intensity, it requires monitoring of a large number of very young children over long periods. An alternative way of estimating the FOI of *Plasmodium* parasites is light microscopic detection of parasites following antimalarial treatment, and this has been applied in field trials of vector control and of vaccines (7, 8).

With the wide acceptance of molecular approaches to malaria epidemiology, more precise measures of molecularly determined FOI ($_{mol}$FOI) can now be generated by genotyping individual parasite infections (9–12). When multiple coinfecting parasite clones within one host can be distinguished, a more realistic measurement of FOI is generated, because natural superinfections can be monitored in hosts who already harbor asymptomatic *P. falciparum* infections (13, 14). PCR-based techniques have the added advantage of higher sensitivity by detecting infections at an earlier time point, when parasite density is still under the limit of detection of microscopy. Because of superinfection, the true FOI is considerably higher than estimates from blood-slide positivity. A molecular approach to estimating FOI therefore provides greater sensitivity.

Whereas the relationship between transmission intensity and the burden of malaria illness is increasingly well described and understood on global (Malaria Atlas Project populations) or continental (African) scales (15, 16), it has been much more difficult to relate local variation in malaria burden to transmission. On the Kenyan coast, locally estimated EIRs were found to be only weakly or not associated with prevalence of infection (17, 18) or incidence of severe disease (17). Except for areas with very low EIR, saturation in the infection rate with increasing transmission was reported from Tanzania and Senegal (19, 20). Such transmission studies at the local level have been hampered by the technical difficulty of accurately quantifying exposure at the population level, and even more at the individual level. Improving on this shortfall, $_{mol}$FOI makes it possible to measure exposure of susceptible hosts and provides data on individuals as well as the study population. We therefore propose to use precise estimates of $_{mol}$FOI to index transmission intensity and to assess effectiveness of malaria control measures. The present study aimed to validate this novel approach in a cohort of young Papua New Guinean children.

Besides $_{mol}$FOI, a second molecular parameter, multiplicity of infection (MOI), is generated by genotyping. MOI is defined as the number of concurrent parasite clones per *P. falciparum*-positive host. In endemic areas, multiple infections of haploid *P. falciparum* clones are the rule, whereby MOI varies according to the degree of malaria endemicity (21). A mean of five concurrent infections was

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found in children from highly endemic settings in Africa (22, 23), whereas in Papua New Guinea (PNG), the reported MOI ranged from 1.7 to 1.9 (24, 25). In children less than 3 y old, each additional infecting clone was associated with a higher prospective risk, whereas for older children it appeared that multiple infections protect against clinical attacks (22, 26–28).

This study was designed to explore patterns in both classical (prevalence of infection and incidence of clinical disease) and molecular measures ($_{mol}$FOI and MOI) of *P. falciparum* burden in children 1–4 y of age and to test for association of $_{mol}$FOI with risk of clinical malaria episodes. A further objective was to determine to what extent variation in $_{mol}$FOI reflects environmental factors, such as seasonality, small-scale geographical variation, effects of insecticide-treated nets (ITNs), and their association with *P. falciparum* episodes.

Results

Between March and September 2006, a total of 264 children aged 0.9–3.2 y (median, 1.7; interquartile range, [1.3, 2.4]) were enrolled and followed for up to 69 wk. Of the children, 93.9% were retained until the end of the study, with between 96.0 and 100.0% of children seen at each scheduled 2-mo blood sampling. A more detailed description of the cohort has been reported previously (29).

Prevalence by PCR and Multiplicity of *P. falciparum* Infections. Over the entire active follow-up period, 87% of children had *msp2* PCR-detectable *P. falciparum* infections at least once during the study period. Prevalence of infection was significantly associated with age (at start of interval) (Table 1), ranging from an average of 25% for children aged 12 mo to 49% for children aged 24–30 mo (Fig. 1*A*). Thereafter, the likelihood of being infected started to level off/decrease, as indicated by the significant negative quadratic coefficient in the final model (Table 1). As in earlier analyses (29), recent antimalarial treatment and the presence of a febrile illness were both associated with increased risk of *P. falciparum* infections (Table 1). Despite adjusting for these factors, prevalence of infection still varied significantly between villages and among children within villages (Table 1, random effects). Prevalence also showed strong seasonal variation (Fig. 1*B* and Table 1), ranging from a low average of 31% in early August to a high of 52% in early February. Personal ITN use (i.e., proportion of nights slept under an ITN) was associated with a very strong reduction in *P. falciparum* prevalence (Fig. 1*C* and Table 1), dropping from an average of 56% with no ITN use to 22% when ITNs were always used.

Unlike prevalence of infection, MOI continued to increase significantly throughout the age range (Fig. 1*A* and Table 1), with mean MOI increasing from about 1.3 at 1 y of age to 2.2 at 4 y of age. Average ITN use was also associated with a reduction in MOI (Fig. 1*C* and Table 1). Average MOI showed only limited, statistically nonsignificant variation over time (Fig. 1*B* and Table 1), and there was no significant variation in MOI between and within villages (Table 1, random effects). Additional parameter estimates are summarized in Table 1.

Force of Infection in 2-mo Intervals. Excluding any period with residual drug levels from the time at risk, the average $_{mol}$FOI was 5.9 (SD 9.6) new *P. falciparum* infections per child per year. The distribution of raw data is shown in Fig. S1. $_{mol}$FOI showed a very pronounced seasonality (Table 1), ranging from a peak of 9.3 in early February to a low of 2.8 at the beginning of August (Fig. 1*B*). Regular ITN use was associated with a significant reduction (Table 1) in acquisition of new clones, from a high of 7.5 with no ITN use to a low of 3.0 (Fig. 1*C*). $_{mol}$FOI appeared to increase linearly between 1 and 3 y of age, with little difference thereafter (Fig. 1*A*). However, there was no significant quadratic term (Table 1 and Fig. S2), despite the apparent leveling off after 3 y of age (Fig. 1*A*). There was highly significant variation in $_{mol}$FOI both between villages and between children living in the same village (Table 1, random effects).

Predictors of Clinical *P. falciparum* Illness. Over the 69 wk of follow-up, a total of 1,134 febrile episodes [incidence rate (IR), 4.60 child per y] with parasitemia (by light microscopy) had been observed (29). *P. falciparum* (Pf) was the most common cause of malarial illness [any density, 630 (IR, 2.56); Pf >2,500/μL, 472 (IR, 1.92)], followed by *P. vivax* (Pv) [any density, 605 (IR, 2.46); Pv >500/μL, 391 (IR, 1.59)]. *P. malariae* and *P. ovale* episodes were rare. All further analyses were done using only the more specific definition of *P. falciparum* malaria of febrile illness plus Pf parasitemia >2,500/μL. Age (at interval), season, ITN use, and recent antimalarial treatment were all significant predictors of clinical episodes of *P. falciparum* malaria (Table 2). The incidence of *P. falciparum* malaria peaked at 3.4 clinical episodes

Table 1. Parameter estimates from GLMMs for prevalence by *msp2* PCR, multiplicity of infection, and force of *P. falciparum* infections

	Prevalence			MOI			$_{mol}$FOI		
	OR	CI_{95}	*P* value	IRR	CI_{95}	*P* value	IRR	CI_{95}	*P* value
Age (at interval)	5.50	[2.10, 14.4]	0.0005	1.17	[1.08, 1.27]	<0.0001	1.38	[1.23, 1.56]	<0.0001
Age^2	0.77	[0.64, 0.93]	0.006						
Season									
Sin (week)	1.37	[1.17, 1.59]	<0.0001*				1.53	[1.37, 1.70]	<0.0001*
Cos (week)	1.42	[1.21, 1.67]					1.58	[1.46, 1.71]	
Year (2007)							0.49	[0.42, 0.59]	<0.0001
ITN use[†]	0.36	[0.24, 0.54]	<0.0001	0.77	[0.67, 0.89]	0.0003	0.49	[0.38, 0.61]	<0.0001
Treated 28 d[‡]	2.17	[1.70, 2.76]	<0.0001						
Fever	1.62	[1.22, 2.16]	0.001				1.22	[1.06, 1.39]	0.004
Random effects									
Village	0.38		<0.0001	~0		0.5	0.05		0.001
Child within village	0.43		<0.0001	~0		0.5	0.22		<0.0001
Seasonal effects									
Amplitude	0.47						0.62		
Peak (week)	6						6		
Trough (week)	32						32		

CI_{95}, 95% confidence interval; IRR, incidence rate ratio; OR, odds ratio.
*Joint *P* value for sin and cos.
[†]Insecticide-treated net use: 0% vs. 100% use.
[‡]Antimalarial treatment within 28 d before the start of the interval.

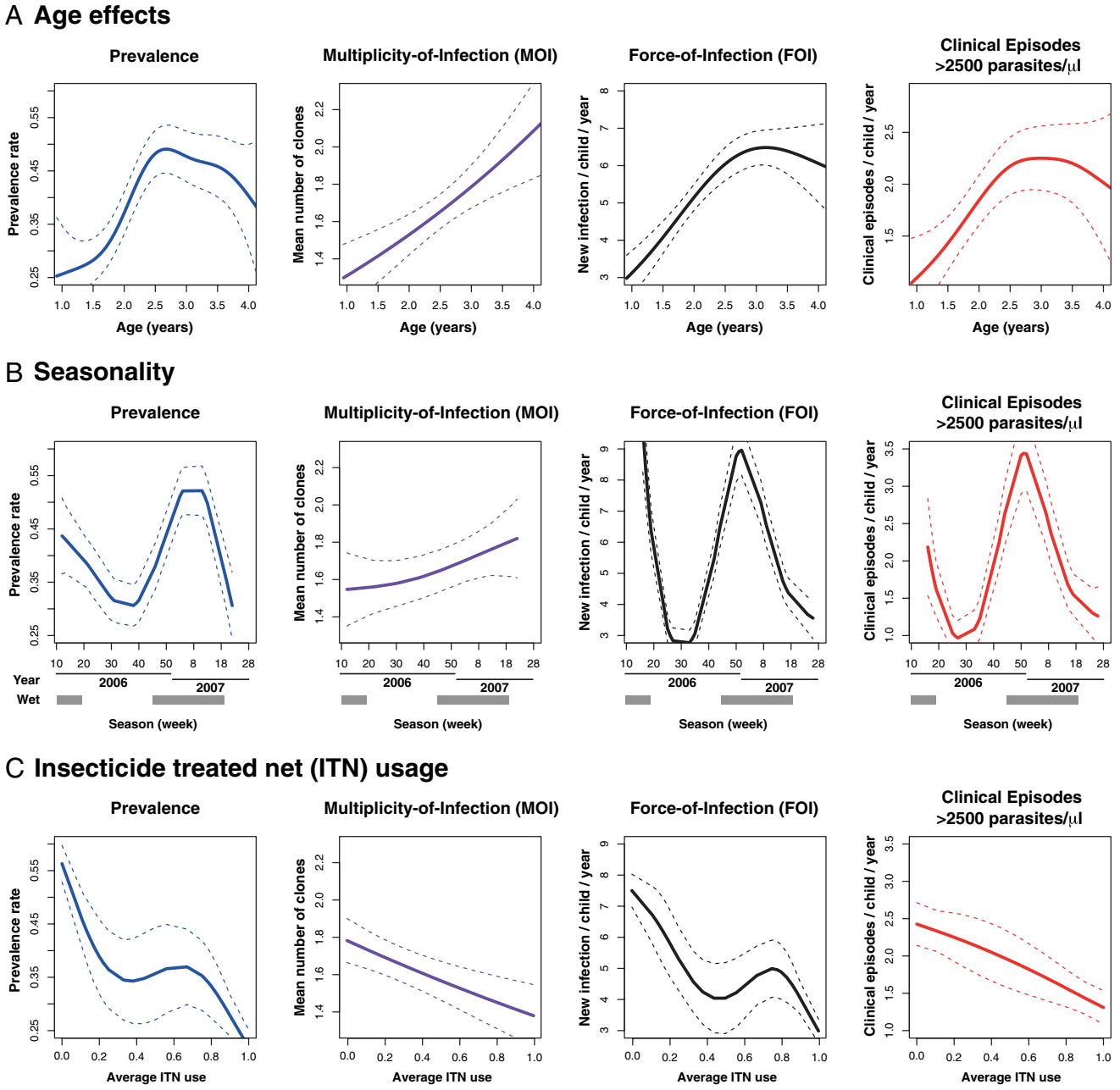

Fig. 1. Patterns of prevalence, MOI, $_{mol}$FOI, and incidence of *P. falciparum* malaria in relation to age, season, and ITN use. Estimated curves from generalized additive models and 95% confidence intervals are shown.

early in the rainy season (early January), and was lowest at 1.0 in the middle of the dry season (early July) (Fig. 1*B*). The incidence of *P. falciparum* malaria increased nonlinearly with age (Fig. 1*A*). Children 3 y of age had an incidence of 2.3, compared with an incidence of 1.0 for 1 y olds (Fig. 1*A*). ITN use also decreased the incidence from 2.4 with no ITN use to 1.3 with constant use (Fig. 1*C* and Table 2). Interestingly, even after adjusting for all of the above factors, the incidence of *P. falciparum* malaria still varied significantly between villages and between children within villages (Table 2, random effects).

When $_{mol}$FOI was added to the model (fitted as the cube root of the rate of new clones acquired per year-at-risk), it was highly significantly associated with an increase in the incidence of *P. falciparum* malaria (Table 2). The incidence of clinical episodes increased rapidly between zero and five new clones per year-at-risk and then steadily increased at a rate of approximately one clinical episode per five newly acquired clones per year-at-risk (Fig. S3). Adjusting for $_{mol}$FOI reduced the influence of all other factors previously associated with incidence of *P. falciparum* malaria (Table 2). The effects of ITN use, age, and recent antimalarial treatment were no longer significantly associated with incidence of *P. falciparum* malaria once $_{mol}$FOI was added to the model. Similarly, the variability between villages and among children within a village no longer contributed significantly after inclusion of $_{mol}$FOI (Table 2). Although significant seasonal variation in incidence remained (Table 2), adjustment for $_{mol}$FOI halved the range of the seasonal variation and shifted the peak of incidence by 6 wk (Fig. 2). Consequently, after taking into account the number of new clones acquired during an interval, the risk of experiencing clinical *P. falciparum* illness was highest at the beginning of the rainy season (i.e., week 48) and lowest at the start of the dry season (week 22). $_{mol}$FOI was therefore the most

Table 2. Parameter estimates for GLMMs predicting the incidence of clinical episodes of *P. falciparum* malaria with density >2,500 parasites/μL with and without FOI

	Base model			$_{mol}$FOI-adjusted		
	IRR	CI_{95}	*P* value	IRR	CI_{95}	*P* value
Age (at interval)	3.34	[1.35, 8.27]	0.009	1.60	[0.69, 3.71]	0.3
Age^2	0.83	[0.69, 0.98]	0.03	0.92	[0.78, 1.08]	0.3
Season						
Sin (week)	1.13	[0.94, 1.34]	<0.0001*	0.89	[0.74, 1.07]	0.0002*
Cos (week)	1.69	[1.48, 1.93]		1.29	[1.13, 1.47]	
Year (2007)	0.73	[0.55, 0.96]	0.03	1.09	[0.83, 1.44]	0.5
ITN use†	0.59	[0.44, 0.79]	0.0004	0.93	[0.73, 1.18]	0.6
FOI‡	—	—	—	2.12	[1.93, 2.31]	<0.0001
Random effects						
Village	0.06		0.005	0.01		0.2
Child within village	0.16		0.005	<0.001		0.5
Log likelihood	−719			−578		
AIC	1,456			1,176		
Seasonal effects						
Amplitude	0.54			0.28		
Peak (week)	2			48		
Trough (week)	28			22		

AIC, Akaike information criterion.
*Joint *P* value for sin and cos.
†Insecticide-treated net use: 0% vs. 100% use.
‡Force of infection (number of new clones per year at risk), cube root-transformed.

important factor significantly associated with risk of clinical *P. falciparum* episodes in the multiple-regression model. Analyses for febrile illness with concurrent parasitemia with any density yielded comparable results (Table S1).

Discussion

Building on earlier descriptive investigations of $_{mol}$FOI, we have now demonstrated the central role of this variable in explaining the epidemiology of *P. falciparum* malaria in young PNG children with low levels of acquired immunity. We conclude that $_{mol}$FOI could serve as a parameter to measure the effect of interventions against malaria. This could be of considerable practical importance in areas of low transmission intensity, where it is increasingly difficult to establish the classical entomological parameters, or where individual or small-scale geographical differences in exposure are important. The global utility of $_{mol}$FOI as an individual measure of transmission needs to be demonstrated by similar cohort studies in different malaria endemicity and other age groups. The ability of $_{mol}$FOI to explain differences in age-specific risk is expected to be restricted to age groups with only limited levels of naturally acquired immunity.

$_{mol}$FOI is particularly useful as an outcome measurement in trials or surveillance measuring the incidence of *P. falciparum* malaria. In children 1–4 y of age, $_{mol}$FOI was very tightly linked with the incidence of clinical disease. Both $_{mol}$FOI and the incidence of clinical disease showed comparable seasonality, increased significantly with age, and were lower in children sleeping under insecticide-impregnated nets. The risk of clinical illness associated with acquiring a new infection was higher at low levels of exposure, but even in children acquiring high numbers of infections, each newly acquired clone continued to increase the risk of clinical disease. This finding is consistent with the overall limited degree of clinical immunity in this cohort.

Moreover, when looking closely at seasonal patterns of $_{mol}$FOI and other parasitological variables, we found that differences in $_{mol}$FOI explained around half of the seasonal variation in *P. falciparum* malaria incidence, an indication that other unmeasured factors significantly contributed to the observed seasonality in episodes. Interestingly, when adjusting for differences in $_{mol}$FOI,

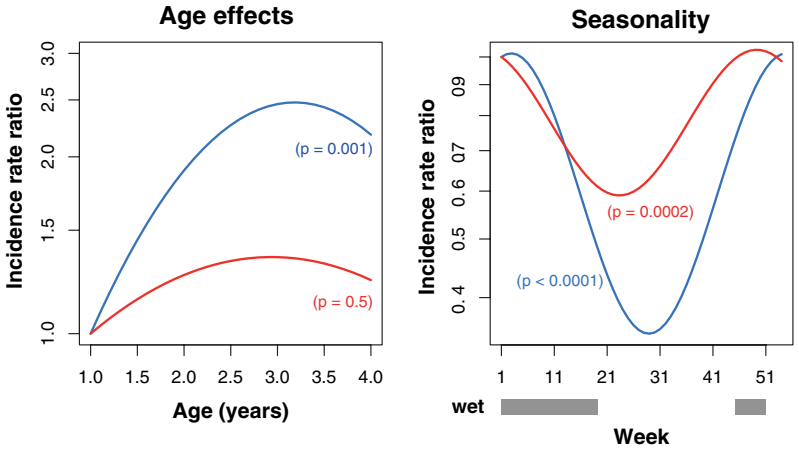

Fig. 2. Incidence rate ratios for age and seasonality on incidence of *P. falciparum* (>2,500/μL) before (blue) and after (red) adjusting for difference in $_{mol}$FOI.

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the peak seasonal risk of *P. falciparum* malaria shifted from the mid to the early rainy season. Infections acquired in the late dry or early rainy season were therefore more likely to lead to clinical illness than those acquired later in the rainy or earlier in the dry season, indicating increasing immunity during the high-transmission season and waning immunity during low transmission. Young children with limited immunity therefore seem to acquire a short-lived protective response during the rainy season, which is supported by findings from a study on the longevity of antibody response to surface antigens that indicated that antibodies decline more rapidly in children <3 y than in older children (30). The latter study, conducted in an area with more pronounced seasonality in The Gambia, also reported seasonal changes in immune status and associated risk of developing clinical malaria. It is therefore important to take into account the overall immune status of the population under surveillance when applying $_{mol}$FOI as a predictor of risk of clinical disease. The current study was conducted in young children with very low levels of clinical immunity against *P. falciparum*. In older subjects with better-established clinical immunity, the association of $_{mol}$FOI with risk of clinical disease is likely to decrease.

The strong, nonlinear increase of $_{mol}$FOI with age may appear surprising, but is well in line with entomological evidence that the number of infective bites received increases with body size (31) and that FOI increased with age in children in the Garki project (32). In addition, PNG vectors are known to readily bite outdoors and in the early hours of the night (33, 34), and older children who are more independent may therefore be at higher risk of being bitten when outdoors. The observed increase in $_{mol}$FOI with age in our cohort was found to almost completely account for the increase in incidence of *P. falciparum* malaria with age.

ITNs both directly limit exposure to mosquitoes of people sleeping under a net and reduce overall transmission by increasing mosquito mortality. A review of The Cochrane Collaboration demonstrated that ITNs reduced the incidence of uncomplicated malarial episodes in areas of stable transmission by 50% compared with no nets (35). A randomized trial of ITNs found a 74% reduction in FOI (measured as time to first infection in infants) (36) corresponding to a 60% reduction in incidence of clinical episodes. We observed a reduction in $_{mol}$FOI of 49% in children always sleeping under a net. The relationship between a reduction in mosquito-biting rates and the resulting changes in $_{mol}$FOI may not be linear, because several parasite clones can be transmitted in a single mosquito bite (37). However, the fact that adjustment for differences in $_{mol}$FOI completely explained the reduction in clinical malaria associated with high ITN use provides a strong confirmation that ITNs do indeed work by reducing individual exposure.

It is well-known from both PNG (33, 34, 38, 39) and elsewhere (40, 41) that malaria transmission can be highly variable even at a small spatial scale. Consequently, it was not surprising that $_{mol}$FOI was also found to show very strong variation both at the levels of villages and individual children living within a village. This microvariability in exposure was also found to be the main determinant of variation of risk of clinical *P. falciparum* disease between and within villages, with other factors such as differences in access to health care or health seeking (42) most likely playing only a minor role in our cohort.

Differences in FOI likely affect prevalence and MOI as well as clinical disease. Therefore, it was not surprising that these parameters showed the same age trend and association with ITN use (Fig. 1*C*). Although all three outcomes showed significant seasonal variation, we found that prevalence peaked earlier in the rainy season (end of January) than MOI (mid February) and $_{mol}$FOI (beginning of March). These differences are more likely a consequence of increased number of clinical episodes, and thus antimalarial treatments later in the rainy season, than represent direct effects of seasonality in exposure.

An essential precondition for accurate estimation of both MOI and $_{mol}$FOI in large field studies is accurate molecular typing. The use of capillary electrophoresis for sizing the highly polymorphic marker gene *msp2* (12, 43, 44) made it possible to precisely track parasite clones in consecutive blood samples, even in large-scale studies with multiple concurrent infections in each sample. The possibility of adapting these techniques to a 96-well format and higher throughput makes it conceivable to routinely apply molecular parameters as outcome measures.

Nevertheless, some parasite clones inevitably remain undetected due to sequestration or densities fluctuating around the detection limit of PCR (24, 45). This imperfect detectability of *P. falciparum* infections can bias estimates of force of infection and should be allowed for, if the intention is to comprehensively quantify parasite dynamics (9, 10, 46). We have tested the effect of detectability in the present study, where two blood samples were collected 24 h apart at all cross-sectional time points and the detectability of a given *msp2* genotype in a single blood sample was estimated at 79% (24). Therefore, we concluded that the sensitivity of detection by PCR does not seem to impair the ability of $_{mol}$FOI to measure differences in exposure or predict clinical incidence. Similarly, the use of additional marker genes would result in the detection of additional strains and thus a higher MOI and $_{mol}$FOI. However, the use of several markers for estimating $_{mol}$FOI entails further statistical challenges because of the need to determine individual haplotypes, making it difficult to quantify MOI or to follow individual haplotypes over time.

Conclusion

Based on our findings of $_{mol}$FOI depicting risk of infection and being very tightly associated with risk of clinical episode, we propose the use of molecular determined FOI as a major parameter in malaria surveillance, for monitoring antimalarial interventions, for example, accompanying sustained and scaled malaria control programs, and for mathematical modeling of the impact of control measures on clinical epidemiology. In the context of malaria vaccine trials, $_{mol}$FOI potentially offers the chance to assess vaccine efficacy in the natural non-drug-treated situation and may help to reduce sample size in clinical trials (14).

Methods

Field Survey and Patients. This study was conducted in a rural area near Maprik, East Sepik Province, Papua New Guinea. A detailed description of the study is given elsewhere (29) and in *SI Methods*. Scientific approval and ethical clearance for the study were obtained from the Medical Research and Advisory Committee of the Ministry of Health in PNG and from the Ethikkommission beider Basel in Switzerland.

Laboratory Procedures. DNA was extracted from cell pellets using a QIAamp 96 DNA Blood Kit (Qiagen) according to the manufacturer's instructions. All samples were genotyped for the polymorphic marker gene merozoite surface protein 2 (*msp2*) by use of capillary electrophoresis for fragment sizing as previously described by Falk et al. (12) with some minor changes and adaptations of PCR conditions for highly purified DNA as described (44). The *msp2* marker was highly polymorphic in the study area, as described previously, with a total of 52 different *msp2* genotypes identified.

Data Analysis. Genotyping will not detect all alleles present in a child if only a blood sample from a single time point is analyzed (24, 45). In the present study, where two samples were collected on 2 consecutive days, we used the combined genotyping data from both days 1 and 2, except for enrollment and final time point, where only one blood sample was collected. Consequently, MOI in each child was defined as the number of different *msp2* genotypes detected at either the day 1 sample alone or (where available) in both samples combined.

$_{mol}$FOI was calculated from the number of new *P. falciparum* infections acquired during 2-mo intervals, expressed as the number of new infections per unit time, and determined by counting all new *msp2* genotypes (i.e., not present in preceding intervals). Samples from regular bleeds as well as from morbidity surveillance were used. Children were considered at risk for acquiring new infections from the first day of an interval, which equals the day following a single or consecutive day bleed. Accordingly, genotypes detected on the first day of each cross-sectional round were considered part of the preceding interval. In line with the pharmacokinetic properties of the drugs (47), children were not considered at risk for 2 wk after treatment with Coartem. The force of infection for each child and interval was subsequently defined as the number of new clones acquired per year-at-risk.

Longitudinal random-effects models or generalized linear mixed models (GLMMs) were used for data analysis (for details, see *SI Methods*). We fit a Poisson-Gaussian random-effects model with a log-link function to relate the fixed and random effects to the number of clinical episodes experienced during a 2-mo interval (defined as febrile illness plus any *P. falciparum* density or *P. falciparum* >2,500 parasites/μL). Similarly, for the prevalence of *P. falciparum* infection by *msp2* PCR, we fit a binomial-Gaussian random-effects model with a logit-link function. Covariates were selected based on earlier analyses of the same data (29). Exploratory data analysis guided the specification of the model when the relationship between the covariates and the outcome did not appear to be linear, such as with age, FOI rate, and season. Seasonality was characterized by two parameters, amplitude and phase, which define a nonlinear regression term. For computational convenience, they are replaced by fixed-phase sine and cosine terms according to ref. 48 (*SI Methods*). For all outcomes except prevalence, an offset was fit to adjust for years at risk. Estimation of these models was done using the lme4 package (49) in R version 2.12 (50).

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