The epidemiology of multiple *Plasmodium falciparum* infections

9. Effect of insecticide-treated bed nets on haemoglobin values, prevalence and multiplicity of infection with *Plasmodium falciparum* in a randomized controlled trial in Tanzania

N. Fraser-Hurt[1*], I. Felger[2], D. Edoh[1], S. Steiger[2], M. Mashaka[1†], H. Masanja[1***], T. Smith[1], F. Mbena[2] and H.-P. Beck[1‡]** *[1]Ifakara Health Research and Development Centre, P.O. Box 53, Ifakara, Tanzania; [2]Swiss Tropical Institute, Socinstrasse 57, 4002 Basel, Switzerland*

Abstract

A randomized controlled trial of insecticide-treated bed nets (ITNs) was conducted in an area of high malaria transmission in Tanzania in order to assess the effects of ITNs on infection and anaemia. One hundred and twenty-two children, aged 5 to 24 months, were randomly allocated to 2 groups, one of which received ITNs. Outcome measures were assessed in 6 consecutive months with monthly cross-sectional surveys. These measures were haemoglobin values, *Plasmodium falciparum* prevalence and density, and multiplicity of infection determined by polymerase chain reaction–restriction fragment length polymorphism analysis (PCR–RFLP) of the *msp2* locus. There was a significant increase in mean heamoglobin values and a significant decrease of 16·4% in microscopically determined *P. falciparum* prevalence in children in the ITN group six months after the start of the trial. Both effects were more pronounced in younger children. However, no significant difference was observed in parasite density or multiplicity of infection among infected children. Comparison with PCR results indicated that microscopically subpatent parasitaemia was more frequently found in children in the ITN group. This, together with the observed similar multiplicity in the 2 groups, suggests that infections are maintained despite ITN use, owing to the chronicity of infections. This study shows that ITNs reduce the risk of anaemia in highly exposed young children. The virtually unchanged multiplicity of infection indicates that the potentially protective concomitant immunity is not compromised.

Keywords: malaria, *Plasmodium falciparum*, multiple infection, insecticide-treated bed nets, genotypes, *msp2* gene, anaemia, immunity, children, Kilombero valley, Tanzania

Introduction

The promotion of insecticide-treated bed nets (ITNs) to reduce exposure to mosquitoes infected with *Plasmodium falciparum* has become a major focus of malaria control options for sub-saharan Africa. Several efficacy trials have given compelling evidence that ITNs improve child survival and reduce malaria-attributable morbidity (ALONSO *et al.*, 1991; D'ALESSANDRO *et al.*, 1995; BINKA *et al.*, 1996; NEVILL *et al.*, 1996; HABLUETZEL *et al.*, 1997). There is, however, concern that if children are protected from infections with ITNs, the development of their malaria-specific immunity might be impaired. There is evidence from studies conducted in both holoendemic and mesoendemic areas that children using a net may have significantly reduced levels of *P. falciparum*-specific immunoglobulin (Ig) G or IgM, when compared to fully exposed children not using a net (GENTON *et al.*, 1994; SNOW *et al.*, 1996). Natural immunity against clinical malaria is believed to develop gradually as a function of frequent infections, and the time period a child is at risk for clinical disease might become longer if the child uses an ITN (SNOW *et al.*, 1994; SNOW & MARSH, 1995; TRAPE & ROGIER, 1996). Hence, a 'rebound effect' might be observed in children who used an ITN during infancy, but later stopped using it and were suddenly highly exposed to infectious mosquitoes.

Recently, we and others have shown that multiple concurrent *P. falciparum* infections, with different genotypes, are associated with a reduced risk of clinical malaria in older children, probably due to the development of premunition (ROBERT *et al.*, 1996; AL-YAMAN *et al.*, 1997; BECK *et al.*, 1997). In a study in Senegal, the force of infection also appeared to be correlated with multiplicity of infection (NTOUMI *et al.*, 1995). Thus, the question arises whether the use of an ITN, assumed to reduce the force of infection, can lead to reduced multiplicity of infections, and thus ultimately compromise the development or maintenance of immunity. Such an effect would be expected to be most pronounced in highly endemic areas, but no randomized controlled ITN trials have yet been conducted in areas of extremely high perennial transmission.

In order to evaluate short-term protective effects in an area of exceptionally high and perennial malaria transmission, we conducted a randomized controlled ITN trial in a village in the Kilombero valley of Tanzania. In addition to assessing effects on anaemia, parasite prevalence and density, multiplicity of infection was determined as a marker for premunition in order to predict possible rebound effects due to reduced exposure. The assumption underlying the study was that, under carefully controlled trial conditions, children in the ITN group would be less exposed to infective mosquito bites than those in the control group.

Study design and Methods

The study took place in the village of Kiberege, Kilombero District, southern Tanzania, which has about 6500 inhabitants and lies at the edge of the Kilombero river plain. In this area, malaria transmission is intense and perennial: the annual entomological inoculation rate is approximately 300 (SMITH *et al.*, 1993) and, by the age of 5 months, more than 60% of children are infected with malaria (KITUA *et al.*, 1996). The Kilombero valley has been the site of various intervention trials, but villagers from Kiberege had not been enrolled in any of these studies.

The present trial started at the end of the rainy season in May 1996, and ended at the peak of the dry season in November 1996. Hence, the recruited children would have received more infectious bites at the beginning of

*Present address: London School of Hygiene and Tropical Medicine, London, UK.
**Present address: Department of Zoology, University of Ghana, Box 67, Legon, Accra, Ghana.
***Present address: AMMP, Box 65243, Dar es Salaam, Tanzania.
†We sincerely regret that Matthew Mashaka died in September 1997, while this paper was in preparation.
‡Author for correspondence; phone +41 61 284 8116, fax +41 61 271 8654, e-mail <beckhp@ubaclu.unibas.ch>

the study than at the end. Children were recruited at the local dispensary when they attended for routine visits as part of the Mother and Child Health (MCH) programme. They were eligible if they were resident in Kiberege, aged 5-24 months, afebrile (axillary temperature <37·5°C), currently not under reported chloroquine treatment, and not using a bed net. Only one child was enrolled from each household. During informed consent taking, the voluntary character of the study, the allocation of nets by chance, and the possibility of early morning check visits were emphasized. In total, 61 children were enrolled in each of the 2 intervention groups, a cohort size which would allow the detection of a 50% reduction in multiplicity between the 2 groups with 90% power and 95% confidence. Children were individually allocated at random in blocks of 4 and allocated to either the 'ITN group' or the 'control group'. The ITNs had been treated with permethrin (0·5 g/m^2) and individually numbered. The nets were also marked with a water-soluble pen, to show whether they had been washed during the trial. The nets were re-treated after 3 months and at the end of the trial, and at the end of the trial the children in the control group also received an ITN.

Initial net installation was done by the parent and checked by project staff, who also checked net use by surprise visits between 05:00 and 08:00. Monthly cross-sectional surveys were conducted during the participants' routine visits to the MCH clinic. At enrolment and at each survey during follow-up, thick blood films were prepared and capillary blood samples collected into ethylenediaminetetraacetic acid Microtainers™ (Becton Dickinson, Rutherford, New Jersey, USA). Haemoglobin concentrations were determined directly (Hemocue™, Ångelholm, Sweden). Fresh urine samples were collected for testing for recent chloroquine consumption. Axillary temperature was measured, and a short morbidity questionnaire was administered in order to record current and past illness, health facility visits and drug consumption. All children presenting with any illness were referred to the health facilities and appropriately treated. All participants carried a medical card, on which prescriptions given at Kiberege dispensary were recorded.

Laboratory procedures

Slides were Giemsa-stained and examined twice, with a third examination if the first 2 were discrepant (ALONSO *et al.*, 1994). Two examinations of 200 fields each of a thick film were made before a slide was declared negative. Chloroquine in urine was measured by thin-layer chromatography (BETSCHART & STEIGER, 1986). Blood samples were stored frozen after plasma separation until a polymerase chain reaction (PCR) was performed. All blood samples, irrespective of the microscopy results, were amplified by PCR for the locus of the merozoite surface protein 2 gene (*msp2*) as described by FELGER *et al.* (1999). All tests with negative results were repeated once, and 10% of all samples were routinely repeated for quality control purposes. PCR products were subjected to an array of restriction enzymes and analysed according to a restriction fragment length polymorphism (RFLP) typing scheme (FELGER *et al.*, 1999).

Statistical methods

Approximate confidence limits for average levels of parasitaemia, parasite density and haemoglobin levels in each follow-up group were calculated without regard to correlations within individuals. For formal statistical testing, the arithmetic mean of all follow-up data for each child was first calculated and the ITN control groups compared using Wilcoxon tests. Parasite densities were log-transformed before analysis. Non-parametric correlations between age at baseline and the response variables also used data first summarized for each child.

Survey, and age-at-survey, adjusted analyses of parasite positivity were carried out using logistic regression models which allowed for correlations within individuals using random-effect terms. These models were fitted using EGRET software (version 2·0; Statistics and Epidemiology Research Corporation, Seattle, Washington, USA).

Results

A total of 122 children was enrolled. Two children were withdrawn during the trial: one child in the control group died during the first month of follow-up, and the parents of one ITN recipient refused to allow follow-up surveys. This left 60 children in each group for analysis. The characteristics of these children at enrolment are shown in Table 1. The mean age was little different in the 2 groups, and the mean haemoglobin levels were virtually the same. There was a small, chance imbalance in the baseline prevalence of *P. falciparum* infections, assessed by microscopy, with a higher prevalence in the control group than in the ITN group (Table 1).

Table 1. Baseline characteristics of the study population

	Insecticide-treated bed net users ITN users	Control group
No. of subjects	60	60
Males (%)	44	54
No. <15 months old	34	30
Age (years)[a]	1·12(0·39)	1·19(0·46)
Haemoglobin level (g/L)[a]	86·5(1·94)	86·9(1·48)
P.falciparum prevalence (%)		
By microscopy	54·1	65·6
By PCR–RFLP	97·8	96·1

[a]Mean (SD in parentheses).

During the 6 cross-sectional dispensary-based surveys, overall compliance was 98%. The homes of children in both groups were visited to assess bed net use, which was reported in 97% of 229 early morning home visits to children in the ITN group. The child was observed sleeping inside or outside the ITN on 82% and 1% of these occasions, respectively. On 17% of visits, direct observation of sleeping behaviour was not possible. In the control group, 14 children were each found once using a torn net or home-made netting but, as the protection provided was negligible, they were not withdrawn from the study. Net washing in between the re-treatment intervals was not observed.

Overall, mean haemoglobin concentrations measured during the follow-up were 93·4 g/L [95% confidence interval (CI) 91·7–95·1] in the ITN group and 88·2 g/L (95% CI 86·6–89·8) in the control group (Wilcoxon's Z=2·51, P=0·012). Haemoglobin values increased in both groups over time, but to a much greater extent in the ITN group (Fig. 1). Haemoglobin concentrations

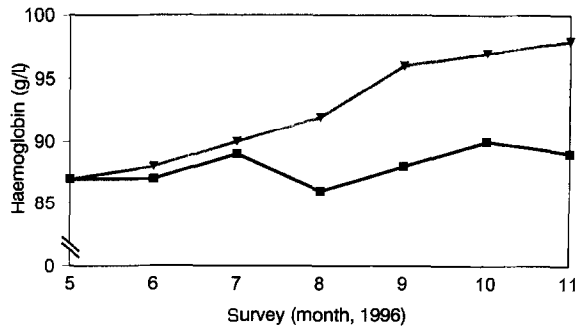

Fig. 1. Haemoglobin values in the group receiving insecticide-treated bed nets (▼) and in the control non-intervention group (■)

during follow-up were not correlated with age at baseline.

Prevalence of *P. falciparum* infections detected by microscopy remained higher in non-ITN users throughout the follow-up period (Fig. 2), with average prevalences of 48% in the ITN group and 65% in the controls. This difference was not due to the imbalance at baseline and indicated that there was still a highly significant difference between the 2 groups in follow-up parasitaemia [likelihood ratio test (LRT): χ^2=6·2, P=0·013]. When assessed by PCR, prevalence of infection at baseline was close to 100% in both groups, implying that 43·7% of children in the ITN group and 30·5% of children in the control group had subpatent parasitaemia at the start of the trial (Table 1). In the course of follow-up, parasite prevalence determined by microscopy decreased in both groups (Fig. 2) (LRT testing effect of survey in a

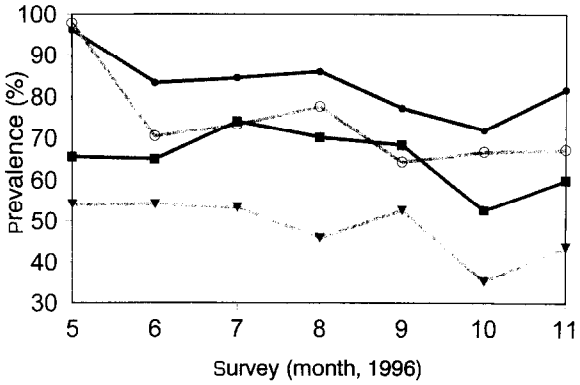

Fig. 2. Point prevalence of *Plasmodium falciparum* in the group receiving insecticide-treated bed nets as determined by microscopy (▼) or by the PCR–RFLP for the *msp2* locus (○) and in the control non-intervention group (■ and ●, respectively).

random effects logistic model: χ^2=10·9, P<0·001), but the decrease in prevalence as determined by PCR was not statistically significant (χ^2=2·8, P=0·093). The prevalence determined by PCR, however, was significantly reduced in the ITN group (LRT: χ^2=4·4, P=0·036).

Children who were aparasitaemic (assessed by microscopy) in any survey, including the baseline, were more common in the ITN group than in the control group (15 vs. 5), and children with parasitaemia in all surveys were found more often in the control group than in the ITN group (19 vs. 10). A similar, but less pronounced, trend was observed when PCR results of infection status were used. In both groups, multiplicity increased with age (Spearman correlation between age at baseline and mean multiplicity: ρ=0·29, P=0·0015).

Bed net use did not appear to lead to a significant change in parasite densities in a comparison of the geometric mean parasite densities of the 2 groups based on microscopically positive films only (Fig. 3, A). Overall, mean parasite densities tended to decrease during the follow-up period. We then tested for differences in densities if subpatent infections were also taken into account. For the purposes of analysis, a density of 10 parasites/μL was arbitrarily allocated to samples which were positive by PCR but microscopically negative. Calculated on this basis, the geometric mean density in follow-up samples from the ITN group was 441 parasites/μL (95% CI 303–643) and in the control group it was 1001 parasites/μL (95% CI 725–1382) (Fig. 3b). The Wilcoxon test indicated that average densities were significantly lower in the ITN group (Z=2·19, P=0·028).

At baseline, multiple *P. falciparum* infections were observed in most of the samples giving positive PCR results, with an average of 2·6 genotypically distinct infections in children allocated to the ITN group and 3·0 infections in children allocated to the control group (Table 2). During follow-up there was a positive correlation between multiplicity in PCR-positive samples and the age at baseline (Spearman's ρ=0·29, P=0·0015), but multiplicity was little affected by season, and no clear difference in average numbers of infections was recorded between the 2 groups (ITN group, mean multiplicity 3·0, 95% CI 2·8–3·2; control group, mean multiplicity 2·9, 95% CI 2·7–3·1) (Table 2). Similarly, no striking difference was found when multiplicity was analysed with respect to the 2 allelic families of *msp2* (data not shown). However, in both

Table 2. Mean multiplicity of *Plasmodium falciparum* infections in the two study groups at baseline and during follow-up

	Survey no.						
	Baseline	1	2	3	4	5	6
Insecticide-treated bed net group	2·56	2·81	3·05	2·56	3·19	3·29	3·08
Control group	3·04	3·12	3·02	2·43	2·82	2·86	3·33

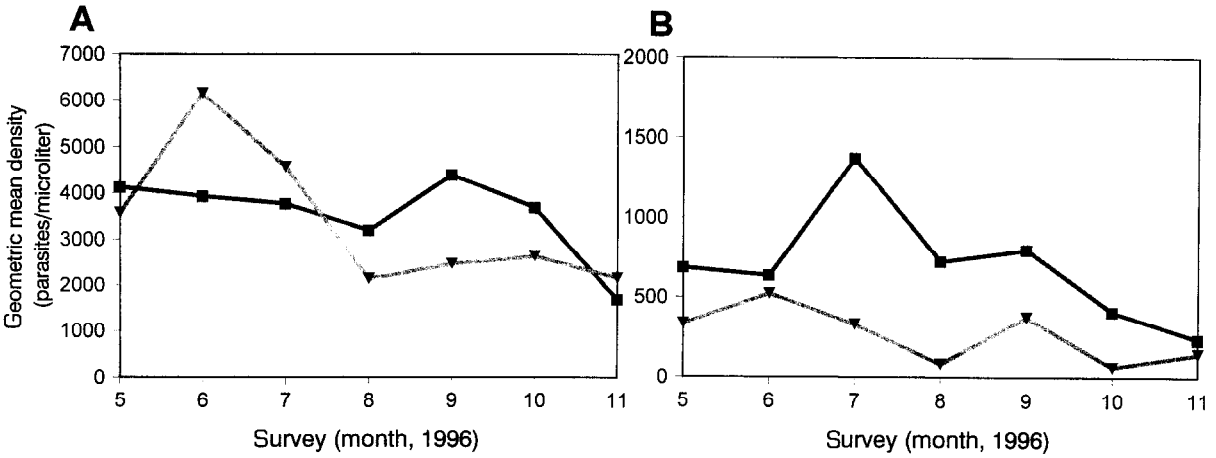

Fig. 3. Geometric mean densities of *Plasmodium falciparum* in (A) microscopically positive samples and (B) all samples positive by PCR–RFLP for the *msp2* locus; microscopically negative samples in this group were assigned an arbitrary density of 10 parasites/μL. ▼, Group receiving insecticide-treated bed nets; ■, control non-intervention group.

groups geometric mean densities determined by microscopy were significantly positively correlated with the number of genotypes observed to be present concurrently (Spearman's $\rho=0{\cdot}36$, $P<0{\cdot}0001$), both with genotypes belonging to the FC27 family ($\rho=0{\cdot}30$, $P<0{\cdot}0001$) or the 3D7 family of alleles ($\rho=0{\cdot}24$, $P<0{\cdot}0001$). Season, age and group status did not affect this association significantly (data not shown).

Axillary temperatures >37·5°C on the day of survey were recorded for 3·2% of the children in the ITN group (total of 349 observations) and for 3·7% of the children in the control group (349 observations). The analysis of average fever rates per child indicated that this difference was not statistically significant (Wilcoxon's $Z=0{\cdot}32$, $P=0{\cdot}8$). Chloroquine concentration was determined in 297 urine samples from the ITN group and 295 samples from the control group. Concentrations >1 mg/mL were found in 20% and 25% of samples, respectively.

Discussion

There is little doubt about the short-term efficacy of ITNs in reducing malarial anaemia in highly endemic areas (MARBIAH *et al.*, 1994; PREMJI *et al.*, 1995; BINKA *et al.*, 1996; HABLUETZEL *et al.*, 1997), parasite prevalence (GRAVES *et al.*, 1987; KARCH *et al.*, 1993; JAENSON *et al.*, 1994), parasite density (KARCH *et al.*, 1993; PREMJI *et al.*, 1995; HABLUETZEL *et al.*, 1997), and acute morbidity and mortality (ALONSO *et al.*, 1991; D'ALESSANDRO *et al.*, 1995; BINKA *et al.*, 1996; NEVILL *et al.*, 1996; HABLUETZEL *et al.*, 1997). Our study has confirmed the efficacy of the nets in controlling high parasite densities and anaemia in an area of very high perennial transmission. The highly significant increase of haemoglobin values in the ITN group is an important finding and is particularly relevant for an area like the Kilombero valley, where anaemia is a leading cause of hospital admissions (SNOW *et al.*, 1994; MENENDEZ *et al.*, 1997).

The question whether ITNs may have a long-term benefit to a host in areas of very intense transmission is still open. No long-term controlled trial has been performed, for obvious ethical reasons, and there is currently a major concern that the use of ITNs might be disappointing in the longer term: instead of reducing the toll taken by malaria, their use may merely lead to an increase in mortality and morbidity in the older age groups (SNOW *et al.*, 1994; SNOW & MARSH, 1995; TRAPE & ROGIER, 1996). Similar doubts have been raised about long-term chemoprophylaxis in endemic areas (GREENWOOD *et al.*, 1995), and data from a recent four-armed intervention trial in Ifakara, Kilombero did indeed indicate a rebound effect on morbidity after stopping chemoprophylaxis (MENENDEZ *et al.*, 1997).

The reason for expecting rebound effects is that partial immunity against malaria develops gradually and is dependent on exposure to infective mosquitoes and the presence and frequency of blood-stage infections. Reduced morbidity is associated particularly with high multiplicity of infections (ROBERT *et al.*, 1996; BECK *et al.*, 1997). In a prospective study in an endemic area of Papua New Guinea, AL-YAMAN *et al.* (1997) showed that individuals with single or no infection had a higher risk of falling ill with malaria subsequently than individuals with multiple clone infections. Furthermore, in a study based on an ecological comparison between sites of different transmission intensity, SNOW and colleagues (1997) showed that severe malaria morbidity was highest in places with intermediate-level exposure.

Because of this evidence for the protective effect of multiplicity of infections, we examined whether the reduction of exposure in children using ITNs had reduced the number of concurrent *P. falciparum* infections in children living in an area of intense transmission, where individuals receive approximately 300 infective bites per year. We observed no reduction in multiplicity during 6 months' use of ITNs, despite the significant reduction in the prevalence of patent *P. falciparum* infections in the children using ITNs. In fact, the multiplicity may have been even higher than observed owing to the technical limitations of the methodology (FELGER *et al.*, 1999). The primary effect of ITNs is to prevent infection, so the observed similarity in microscopically-assessed parasite densities of patent infections in the 2 groups indicated that the use of nets did not affect the course of a patent infection once it had been established, a finding also reported by GENTON *et al.* (1994).

Though microscopy indicated a reduction of parasite prevalence, data from the PCR, which can detect subpatent parasitaemia, indicated that ITNs had only a small effect on prevalence, but that mean parasite densities were significantly reduced in ITN users. From our data we assume that, in children above one year of age, *P. falciparum* infections, once established, may become chronic and last for a long time at low densities (SMITH *et al.*, 1999a). The fact that subpatent low-density infections were frequently observed in ITN users suggests that such chronic infections form a large proportion of all infections. If this is the case limited reduction in exposure will not have much impact on multiplicity. However, preventing only a small proportion of infections may not hinder the establishment of premunition (SMITH *et al.*, 1999b), which might protect against high parasite densities resulting from new inoculations which would otherwise contribute disproportionately to the parasite load and hence to clinical malaria and anaemia.

In order to look more closely at possible explanations for the similarity in multiplicity between ITN and control groups, we carried out further longitudinal analyses of the PCR–RFLP data, which are presented in a companion paper (SMITH *et al.*, 1999a). From these analyses and from the data presented here, we concluded that it is unlikely that there will be a reduction of multiplicity of infection due to the use of ITNs even over extended periods, provided further infections occur to maintain the multiplicity and premunition. In such a situation the burden of morbidity from frequent high-density infections would be reduced.

These results should be confirmed by studies with longer observation periods and in areas of different endemicity. It should also be emphasized that we have analysed the effect of ITNs on children aged over 5 months of age, and the situation may be quite different in younger infants where little specific immunity develops against infections.

In conclusion, the results of this study provide evidence for the beneficial effect of ITNs in highly endemic areas such as our study site, where anaemia is the major cause of morbidity, and suggest that a major rebound effect need not be expected. The substantial increase in haemoglobin level and the persistence of multiplicity found in our study support this view. Even if morbidity were to be shifted towards older age groups, a more mature immune system and a more stable physiology might protect an older child from the most severe forms of malaria morbidity in highly endemic areas such as parts of Tanzania (MOLINEAUX, 1997). None the less, careful immunoparasitological and epidemiological monitoring should accompany any large-scale implementation of ITNs as control measures.

Acknowledgements

We are grateful to the children and their guardians for their participation in this study, to the field and laboratory staff of the Ifakara Health Research and Development Centre, and to J. Armstrong Schellenberg and M. Tanner for critical comments on the manuscript. Research clearance, including ethical clearance for this study, was given by the Medical Research Co-ordination Committee of the National Institute of Medical Research through the Tanzanian Commssion for Science and Technology (reference no. NSR/RCA 90). The laboratory investigations were supported by the Friedrich Miescher Insti-

tute, Basel. The field component of the study was financially supported by the Rotary Club Switzerland District 1980, and the laboratory component was supported by Swiss National Foundation grant no. 3200-045616.95/1.

References

Alonso, P. L., Lindsay, S. W., Armstrong, J. R., Conteh, M., Hill, A. G., David, P. H., Fegan, G., de Francisco, A., Hall, A. J., Shenton, F. C., Cham, K. & Greenwood, B. M. (1991). The effect of insecticide-treated bed nets on mortality of Gambian children. *Lancet*, **337**, 1499–1502.

Alonso, P. L., Smith, T., Armstrong Schellenberg, J. R. M., Masanja, H., Mwankusye, S., Urassa, H., Bastos de Azevedo, I., Chongela, J., Kobero, S., Menendez, C., Hurt, N., Thomas, M. C., Lyimo, E., Weiss, N. A., Hayes, R., Kitua, A. Y., Lopez, M. C., Kilama, W. L., Teuscher, T. & Tanner, M. (1994). Randomised trial of efficacy of the SPf66 vaccine against *Plasmodium falciparum* malaria in children in southern Tanzania. *Lancet*, **344**, 1175–1181.

Al-Yaman, F., Genton, B., Reeder, J., Anders, R., Smith, T. & Alpers, M. P. (1997). Reduced risk of clinical malaria in a highly endemic area in children infected with multiple clones of *Plasmodium falciparum*: a prospective community study. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, **91**, 602–605.

Beck, H.-P., Felger, I., Huber, W., Steiger, S., Smith, T., Weiss, N., Alonso, P. L. & Tanner, M. (1997). Analysis of multiple *Plasmodium falciparum* infections in Tanzanian children during the trial of the malaria vaccine SPf66. *Journal of Infectious Diseases*, **175**, 921–926.

Betschart, B. & Steiger, S. (1986). Quantitative determination of chloroquine and desethylchloroquine in biological fluids by high performance thin layer chromatography. *Acta Tropica*, **43**, 125–130.

Binka, F. N., Kubaje, A., Adjuik, M., Williams, L. A., Lengeler, C., Maude, G. H., Armah, G. E., Kajihara, B., Adiamah, J. H. & Smith, P. G. (1996). Impact of permethrin-impregnated bednets on child mortality in Kassena–Nankana District, Ghana: a randomized controlled trial. *Tropical Medicine and International Health*, **1**, 147–154.

D'Alessandro, U. D., Olaleye, B. O., McGuire, W., Langerock, P., Bennett, S., Aikins, M. K., Thomson, M. C., Cham, M. K., Cham, B. A. & Greenwood, B. (1995). Mortality and morbidity from malaria in Gambian children after introduction of an impregnated bednet programme. *Lancet*, **345**, 479–483.

Felger, I., Irion, A., Steiger, S. & Beck, H.-P. (1999). The epidemiology of multiple *Plasmodium falciparum* infections 2. Genotypes of merozoite surface protein 2 of *Plasmodium falciparum* in Tanzania. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, **93**, supplement 1, S1//3–S1/9.

Genton, B., Hii, J., Al-Yaman, F., Paru, R. & Beck, H.-P. (1994). The use of untreated bednets and malaria infection, morbidity and immunity. *Annals of Tropical Medicine and Parasitology*, **88**, 263–270.

Graves, P. M., Brabin, B. J., Charlwood, J. D., Burkot, T. R., Cattani, J. A., Ginny, M., Paino, J., Gibson, F. D. & Alpers, M. P. (1987). Reduction in incidence and prevalence of *Plasmodium falciparum* in under-5-year-old children by permethrin impregnation of mosquito nets. *Bulletin of the World Health Organization*, **65**, 869–877.

Greenwood, B. M., David, P. H., Otoo-Forbes, L. N., Allen, S. J., Alonso, P. L., Armstrong Schellenberg, J. R., Byass, P., Hurwitz, M., Menon, A. & Snow, R. W. (1995). Mortality and morbidity from malaria after stopping malaria chemoprophylaxis. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, **89**, 629–633.

Habluetzel, A., Diallo, D. A., Esposito, F., Lamizana, L., Pagnoni, F., Lengeler, C., Traor, J. C. & Cousens, S. N. (1997). Do insecticide-treated curtains reduce all-cause child mortality in Burkina Faso? *Tropical Medicine and International Health*, **23**, 855–862.

Jaenson, T. G., Gomes, M. J., Barreto dos Santos, R. C., Petrarca, V., Fortini, D., Evora, J. & Crato, J. (1994). Control of endophagic *Anopheles* mosquitoes and human malaria in Guinea Bissau, West Africa by permethrin-treated bed nets. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, **88**, 620–624.

Karch, S., Garin, B., Asidi, N., Manzambi, Z., Salaun, J. J. & Mouchet, J. (1993). Mosquito nets impregnated against malaria in Zaire. *Annales de la Société Belge de Médecine Tropicale*, **73**, 37–53.

Kitua, A. Y., Smith, T., Alonso, P. L., Masanja, H., Menendez, C., Urassa, H., Kimario, J. & Tanner, M. (1996). *Plasmodium falciparum* malaria in the first year of life in an area of intense and perennial transmission. *Tropical Medicine and International Health*, **1**, 475–484.

Marbiah, N. T., Magbiti, E., Lines, J. D., Maude, G. H., Greenwood, B. M., Bradley, D. & Petersen, E. (1994). A double comparative study of the acceptability of untreated bed nets versus permethrin, lambda-cyhalothrin and deltamethrin impregnated bed nets. *Memorias do Instituto Oswaldo Cruz*, **89**, supplement 2, 3-7.

Menendez, C., Kahigwa, E., Hirt, R., Vounatsou, P., Font, F., Aponte, J., Acosta, C. J., Schellenberg, D., Galindo, C. M., Kimario, J., Masanja, H., Urassa, H., Fumado, V., Brabin, B., Smith, T., Kitua, A. Y., Tanner, M. & Alonso, P. L. (1997). Randomised placebo-controlled trial of iron supplementation and malaria chemoprophylaxis for the prevention of severe anaemia and malaria in Tanzanian infants. *Lancet*, **350**, 844–850.

Molineaux, L. (1997). Nature's experiment: what implications for malaria prevention? *Lancet*, **349**, 1636–1637.

Nevill, C. G., Some, E. S., Mung'ala, V. O., Mutemi, W., New, L., Marsh, K., Lengeler, C. & Snow, R. W. (1996). Insecticide-treated bednets reduce mortality and severe morbidity from malaria among children on the Kenyan coast. *Tropical Medicine and International Health*, **1**, 139–146.

Ntoumi, F., Contamin, H., Rogier, C., Bonnefoy, S., Trape, J. F. & Mercereau-Puijalon, O. (1995). Age-dependent carriage of multiple *Plasmodium falciparum* merozoite surface antigen-2 alleles in asymptomatic malaria infections. *American Journal of Tropical Medicine and Hygiene*, **52**, 81–88.

Premji, Z., Lubega, P., Hamisi, Y., Mchopa, E., Minjas, J., Checkley, W. & Shiff, C. (1995). Changes in malaria associated morbidity in children using insecticide treated mosquito nets in the Bagamoyo district of coastal Tanzania. *Tropical Medicine and Parasitology*, **46**, 147–153.

Robert, F., Ntoumi, F., Angel, G., Candito, D., Rogier, C., Fandeur, T., Sarthou, J. L. & Mercereau-Puijalon, O. (1996). Extensive genetic diversity of *Plasmodium falciparum* isolates collected from patients with severe malaria in Dakar, Senegal. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, **90**, 704–711.

Smith, T., Charlwood, J. D., Kihonda, J., Mwankusye, S., Billingsley, P., Meuwissen, J., Lyimo, E., Takken, W., Teuscher, T. & Tanner, M. (1993). Absence of seasonal variation in malaria parasitaemia in an area of intense seasonal transmission. *Acta Tropica*, **54**, 55–72.

Smith, T., Felger, I., Fraser-Hurt, N. & Beck, H.-P. (1999a). The epidemiology of multiple *Plasmodium falciparum* infections 10. Effects of insecticide-treated bed nets on the dynamics of multiple *Plasmodium falciparum* infections. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, **93**, supplement 1, S1/53–S1/57.

Smith, T., Felger, I., Tanner, M. & Beck, H.-P. (1999b). The epidemiology of multiple *Plasmodium falciparum* infections 11. Premunition in *Plasmodium falciparum* infections: insights from studies of multiple infections. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, **93**, supplement 1, S1/59–S1/64.

Snow, R. W. & Marsh, K. (1995). Will reducing *Plasmodium falciparum* transmission alter malaria mortality among African children? *Parasitology Today*, **11**, 188–190.

Snow, R. W., Bastos de Azevedo, I., Lowe, B. S., Kabiru, E. W., Nevill, C. G., Mwankusye, S., Kassiga, G., Marsh, K. & Teuscher, T. (1994). Severe childhood malaria in two areas of markedly different falciparum transmission in East Africa. *Acta Tropica*, **57**, 289–300.

Snow, R. W., Molyneux, C. S., Warn, P. A., Omumbo, J., Nevill, C. G., Gupta, S. & Marsh, K. (1996). Infant parasite rates and immunoglobulin M seroprevalence as a measure of exposure to *Plasmodium falciparum* during a randomized controlled trial of insecticide-treated bed nets on the Kenyan coast. *American Journal of Tropical Medicine and Hygiene*, **55**, 144–149.

Snow, R. W., Omumbo, J. A., Lowe, B., Molyneux, C. S., Obiero, J. O., Palmer, A., Weber, M. W., Pinder, M., Nahlen, B., Obonyo, C., Newbold, C., Gupta, S. & Marsh, K. (1997). Relation between severe malaria morbidity in children and level of *Plasmodium falciparum* transmission in Africa. *Lancet*, **349**, 1650–1654.

Trape, J.-F. & Rogier, C. (1996). Combating malaria morbidity and mortality by reducing transmission. *Parasitology Today*, **12**, 236–240.