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1 **Treatment of Chronic Asymptomatic *Plasmodium falciparum* Infection Does Not Increase the**  
2 **Risk of Clinical Malaria upon Reinfection**

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38 [Key Points:](#)

39 Chronic asymptomatic *P. falciparum* infection during the dry season predicts decreased clinical  
40 malaria risk during the ensuing malaria season; however, treating these infections did not alter this  
41 reduced risk, challenging the notion that chronic *P. falciparum* infection maintains malaria immunity.

42

43

44 **Abstract**

45 **Background** Chronic asymptomatic *Plasmodium falciparum* infections are common in endemic areas  
46 and are thought to contribute to the maintenance of malaria immunity. Whether treatment of these  
47 infections increases the subsequent risk of clinical episodes of malaria is unclear.

48 **Methods** In a three-year study in Mali, asymptomatic individuals with or without *P. falciparum*  
49 infection at the end of the six-month dry season were identified by PCR, and clinical malaria risk was  
50 compared during the ensuing six-month malaria transmission season. At the end of the second dry  
51 season, three groups of asymptomatic children were identified: 1) children infected with *P. falciparum*  
52 as detected by rapid diagnostic testing (RDT) who were treated with antimalarials (n=104), 2) RDT-  
53 negative children whose untreated *P. falciparum* infections were detected retrospectively by PCR  
54 (n=55), and 3) uninfected children (RDT/PCR negative) (n=434). Clinical malaria risk during two  
55 subsequent malaria seasons was compared. *P. falciparum*-specific antibody kinetics during the dry  
56 season were compared in children who did or did not harbour asymptomatic *P. falciparum* infections.

57 **Results** Chronic asymptomatic *P. falciparum* infection predicted decreased clinical malaria risk  
58 during the subsequent malaria season(s); treatment of these infections did not alter this reduced risk. *P.*  
59 *falciparum*-specific antibodies declined similarly in children who did or did not harbour chronic  
60 asymptomatic *P. falciparum* infection during the dry season.

61 **Conclusions** These findings challenge the notion that chronic asymptomatic *P. falciparum* infection  
62 maintains malaria immunity and suggest that mass drug administration during the dry season should  
63 not increase the subsequent risk of clinical malaria.

64 **Introduction**

65  
66 *Plasmodium falciparum* is responsible for ~200 million cases of malaria and 400,000 deaths annually  
67 [1]. Encouragingly, the scale up of mosquito control measures and artemisinin-based combination  
68 therapy has been associated with reduced malaria burden in many regions [2]. Consequently, an  
69 increasing number of endemic countries are working towards elimination and considering the  
70 interventions that will be required to achieve this objective. Importantly, a large proportion of people  
71 in endemic areas are infected with *P. falciparum* without symptoms [3, 4]. This clinically silent  
72 parasite reservoir, which persists for months to years [5] and contributes to ongoing malaria  
73 transmission [4, 6-9], poses a challenge for elimination efforts. The strategies of antimalarial mass  
74 drug administration (MDA) to at risk populations or mass screening and treatment (MSAT) of  
75 asymptotically infected individuals are being considered in certain settings [10-12], particularly in  
76 areas of seasonal transmission where MDA during the dry season could reduce the number of  
77 gametocyte carriers and decrease transmission to the mosquito vector as the rainy season ensues [11,  
78 13, 14].

79  
80 However, asymptomatic *P. falciparum* infections have long been thought to directly contribute to the  
81 maintenance of immunity to malaria, a notion referred to as ‘premunition’ [15-17]. Consistent with  
82 this hypothesis, studies in areas of seasonal malaria have shown that asymptomatic *P. falciparum*  
83 infection at the end of the dry season predicts decreased risk of febrile malaria during the ensuing  
84 malaria season [18-22]. This raises the question of whether treatment of asymptomatic infections  
85 during the dry season might increase the risk of symptomatic malaria in the event of *P. falciparum* re-  
86 infection.

87  
88 Two studies in areas of seasonal malaria assessed the impact of treating asymptomatic *P. falciparum*  
89 infection during the dry season on the subsequent risk of clinical malaria. A trial in the Gambia  
90 randomized villages to placebo versus one dose of sulfadoxine-pyrimethamine combined with one  
91 dose of artesunate and found no difference in malaria incidence during 20 weeks of follow-up,

92 although there was a significant drop in the incidence of malaria during the period immediately after  
93 drug administration [23]. Similarly, a trial in Burkina Faso randomized villages to screening and  
94 treatment of asymptomatic *P. falciparum* infection with artemether-lumefantrine (AL) or no  
95 intervention and found no difference in the subsequent incidence of malaria [24]. In contrast, a study  
96 in the Zambia—where malaria transmission is year-round—randomized health districts during the low  
97 transmission season to screening and treatment of infected individuals with AL or no intervention and  
98 found a modest decrease in malaria risk in the intervention group [25].

99

100 Importantly, these studies compared malaria risk at the community rather than individual level, which  
101 may have confounded the results due to heterogeneity in *P. falciparum* transmission across  
102 communities. Additionally, at the time of treatment these studies did not distinguish chronic  
103 asymptomatic *P. falciparum* infection from recently transmitted infections that may have progressed  
104 to clinical malaria without treatment [26], and which may have different effects on host immunity.  
105 Together, the limitations of these studies leave open the question of whether treatment of chronic  
106 asymptomatic *P. falciparum* infection impacts the subsequent risk of clinical malaria at the individual  
107 level.

108

109 In this longitudinal study conducted in an area of seasonal malaria we addressed four objectives: 1)  
110 determine whether asymptomatic *P. falciparum* parasitemia detected at the end of the six-month dry  
111 season represents chronic infection, 2) confirm that asymptomatic *P. falciparum* infections during the  
112 dry season predict protection from clinical malaria during the ensuing malaria season, 3) determine the  
113 impact of treating asymptomatic *P. falciparum* infection during the dry season on the subsequent risk  
114 of clinical malaria, and 4) determine whether chronic asymptomatic *P. falciparum* infection maintains  
115 *P. falciparum*-specific humoral immunity.

116

## 117 **METHODS**

118

### 119 **Ethics statement**

|

120 The Ethics Committee of the FMPOS at the University of Bamako, and the NIAID/NIH IRB approved  
121 this study. Written informed consent was obtained from all subjects and the parents/guardians of  
122 participating children. The study is registered on <http://www.clinicaltrials.gov> (NCT01322581).

123

#### 124 **Study design and participants**

125 From May 2011 through December 2013 a cohort study was conducted in Kalifabougou, Mali, a rural  
126 village of ~5000 inhabitants where malaria transmission occurs from July through December. A single  
127 clinic and pharmacy provided the only access to antimalarial drugs. A detailed description of the study  
128 site and cohort design has been published elsewhere [27]. From an age-stratified, random sample of  
129 the entire village population, 695 healthy individuals aged six months to 25 years were enrolled.

130 Exclusion criteria were haemoglobin concentration  $<7$  g/dL, axillary temperature  $\geq 37.5^{\circ}\text{C}$ , acute  
131 systemic illness, or use of antimalarial or immunosuppressive medications in the preceding 30 days.

132 Clinical malaria episodes were detected prospectively by active and passive surveillance and were  
133 defined by an axillary temperature of  $\geq 37.5^{\circ}\text{C}$ ,  $\geq 2500$  asexual parasites/ $\mu\text{L}$  of blood, and no other  
134 cause of fever on physical examination.

135

#### 136 **Detection of *P. falciparum* infection**

137 Thick blood smears were stained with Giemsa and *Plasmodium* parasites were counted against 300  
138 leukocytes; parasite densities were recorded as the number of parasites/ $\mu\text{L}$  of whole blood based on a  
139 mean leukocyte count of 7500 cells/ $\mu\text{L}$ . Two expert microscopists evaluated each smear separately,  
140 and a third resolved discrepancies. The First Response® Combo Malaria Ag (pLDH/HRP2) card was  
141 used as a rapid diagnostic test (RDT), the sensitivity of which is  $\sim 100$  parasites/ $\mu\text{L}$  [28]. Nested PCR  
142 amplification of *Plasmodium* DNA was performed from dried blood spots as previously described  
143 [27], the sensitivity of which is  $\sim 0.5 - 1$  parasites/ $\mu\text{L}$  [27].

144

145 Additional methods are described online in supplementary information.

146

#### 147 **Results**

|

148

149 **Asymptomatic *P. falciparum* infection during the dry season is associated with lower risk of**  
150 **clinical malaria during the ensuing malaria season**

151

152 We sought to confirm prior studies that associated asymptomatic *P. falciparum* infection with lower  
153 risk of clinical malaria [18, 19, 21, 22]. During a two-week period in May 2011 we enrolled 695  
154 asymptomatic subjects just prior to the 6-month malaria season (Figure 1A). The prevalence of *P.*  
155 *falciparum* infection at enrolment was 45.6% by PCR and 26.3% by blood smear. The prevalence of  
156 infection by PCR plateaued by 8 years of age, while the prevalence by blood smear declined after 8  
157 years of age (Figure 1B), consistent with an age-dependent decrease in parasitemia [27].

158

159 During the ensuing six-month malaria season, clinical malaria episodes were detected by weekly  
160 active surveillance and self-referral. Consistent with prior studies [18-22], asymptomatic *P.*  
161 *falciparum* infection at the end of the dry season was associated with lower risk of febrile malaria  
162 during the ensuing malaria season ( $p < 0.0001$ ; Figure 1C), an association that remained significant  
163 after adjusting for age, gender and hemoglobin (Hb) type ( $P < 0.0001$ ; Figure 1D). The risk of febrile  
164 malaria was not significantly different between PCR<sup>+</sup>smear<sup>+</sup> subjects and PCR<sup>+</sup>smear<sup>-</sup> subjects  
165 ( $p = 0.51$ ; Figure 1C), indicating that the difference in baseline parasitemia between these groups did  
166 not affect subsequent malaria risk. Hereafter, all analyses focus on children  $\leq 11$  years of age—the age  
167 group that experiences the majority of clinical malaria episodes in this cohort [27].

168

169 **Treatment of chronic asymptomatic *P. falciparum* infection does not change the risk of clinical**  
170 **malaria during the subsequent malaria season**

171

172 The association between asymptomatic *P. falciparum* infection during the dry season and subsequent  
173 protection from febrile malaria suggests that treatment of asymptomatic infection at the end of the dry  
174 season could increase the risk of clinical malaria during the ensuing malaria season. To test this  
175 hypothesis, we screened the same cohort for *P. falciparum* infection (all asymptomatic) at the end of

|



176 the second dry season (May 2012) using an RDT with a sensitivity comparable to blood smear [29].  
177 All subjects found to be *P. falciparum*-infected by RDT (n=104) were treated with a standard 3-day  
178 course of AL, the first daily dose of which was directly observed by study staff. Dried blood spots  
179 collected from RDT<sup>-</sup> subjects at the same timepoint (n=489) were later analyzed by PCR to  
180 retrospectively identify two additional groups that did not receive antimalarials: RDT<sup>-</sup>PCR<sup>+</sup> subjects  
181 (n=55) and RDT<sup>-</sup>PCR<sup>-</sup> subjects (n=434).  
182  
183 Because asymptomatic *P. falciparum* infections detected during cross-sectional surveys can become  
184 symptomatic within days or weeks of initial detection [3, 4, 30], we sought to confirm that *P.*  
185 *falciparum* infections detected at the end of the dry season in May 2012 were chronic and  
186 asymptomatic. We found that subjects infected with *P. falciparum* in May 2012 were highly likely to  
187 have been infected at the start of the dry season (January 2012) [OR: 842.6 c.i. (200.2, 3546.3), P <  
188 0.0001]; and through the mid-dry season (March 2012) [OR: 172.9 c.i. (79.8, 374.5), P < 0.0001]; and  
189 conversely, uninfected subjects at the start of the dry season remained uninfected at the end of the dry  
190 season (Supplementary Figure 1). During the same time period, no cases of clinical malaria were  
191 detected. We also examined parasites collected in January and May 2012, from individuals who tested  
192 PCR<sup>+</sup> in May 2012, for 6 microsatellite loci previously used to characterize the genetic diversity of *P.*  
193 *falciparum* [31], and we obtained low F<sub>st</sub> values between the populations (F<sub>st</sub> January vs. May = 0.004),  
194 indicating that the two populations were genetically very similar (Figure 2A). Additionally, analysis of  
195 the polymorphic region of the *P. falciparum* msp2 locus in January and May 2012 indicated that  
196 asymptomatic infections were polyclonal at both timepoints and 47% of subjects harboured at least  
197 one common parasite clone at both timepoints (Figure 2B). Together with our observation that the  
198 entomological inoculation rate is near zero during the dry season, these data indicate that  
199 asymptomatic *P. falciparum* infections detected at the end of the dry season had persisted as chronic  
200 asymptomatic infections throughout the preceding dry season.  
201  
202 The characteristics of the three groups defined in May 2012 (RDT<sup>+</sup> treated, RDT<sup>-</sup>PCR<sup>+</sup> untreated, and  
203 RDT<sup>-</sup>PCR<sup>-</sup> untreated) are shown in Table 1. Consistent with the first year of the study, the RDT<sup>-</sup>PCR<sup>-</sup>

204 group had the highest risk of febrile malaria during the second malaria season (Figure 3A), while  
205 febrile malaria risk in the RDT<sup>+</sup> treated and RDT<sup>-</sup>PCR<sup>+</sup> untreated groups was similar in both  
206 univariate (Figure 3A) and multivariate analyses (Figure 3B). We observed the same pattern during  
207 the third malaria season (Figure 3C and 3D), indicating that treatment of chronic asymptomatic *P.*  
208 *falciparum* infection at the end of the dry season does not change the risk of clinical malaria during  
209 two subsequent malaria seasons.

210

### 211 ***P. falciparum*-specific humoral immunity decreases similarly with or without chronic** 212 **asymptomatic infection**

213

214 Because treatment of asymptomatic *P. falciparum* infection at the end of the dry season did not  
215 increase the subsequent risk of febrile malaria, we hypothesized that chronic asymptomatic *P.*  
216 *falciparum* infection *per se* does not maintain malaria immunity, but is instead a marker of higher past  
217 *P. falciparum* exposure and thus higher cumulative immunity. We tested this hypothesis by comparing  
218 antibody responses to 862 *P. falciparum* proteins before and after the dry season in age-matched  
219 children who did or did not harbour asymptomatic *P. falciparum* over the same time period. At both  
220 timepoints the breadth and magnitude of *P. falciparum*-specific antibodies were higher in subjects  
221 who carried parasites through the dry season (Figure 4A-C); however, both antibody breadth and  
222 magnitude decreased similarly during the dry season in infected and uninfected subjects (Figures 4D  
223 and 4E), suggesting that chronic asymptomatic *P. falciparum* infection *per se* does not contribute  
224 significantly to the maintenance of humoral immunity to malaria.

225

### 226 **Discussion**

227

228 Here we investigated the impact of treating chronic asymptomatic *P. falciparum* at the end of the dry  
229 season on the subsequent risk of clinical malaria. In doing so we tested the long-standing hypothesis  
230 that asymptomatic *P. falciparum* infection maintains immunity to malaria [15-17, 32, 33]. We found  
231 that treatment of asymptomatic *P. falciparum* infection at the end of the dry season did not increase

|

232 clinical malaria risk at the individual level during two subsequent malaria seasons. Moreover, *P.*  
233 *falciparum*-specific antibodies declined at a similar rate in children who did or did not harbour  
234 asymptomatic *P. falciparum* over the dry season. Together these findings challenge the notion that  
235 asymptomatic *P. falciparum* infection maintains clinical and humoral immunity to malaria and suggest  
236 that MDA during the dry season should not increase the subsequent risk of clinical malaria at the  
237 individual level. In contrast, seasonal malaria chemoprevention (SMC) [34] - which prevents the  
238 progression of new blood-stage infections during the transmission season - has been associated in  
239 some studies with increased malaria risk (i.e. rebound) after discontinuation of SMC [35, 36].  
240 Therefore, we hypothesize that recently transmitted *P. falciparum* parasites more effectively induce  
241 immune responses relative to parasites that have persisted in blood for several months during the dry  
242 season. A differential capacity to trigger host immune responses could reflect epigenetic,  
243 transcriptional and metabolic differences between newly transmitted parasites and parasites that persist  
244 during long periods of asexual replication in blood.

245  
246 We found that the breadth and magnitude of IgG specific for 862 *P. falciparum* proteins/polypeptides  
247 declined at a similar rate in children who did or did not carry asymptomatic *P. falciparum* infection  
248 during the dry season. Similarly, Consistent with our antibody data, a study comparing Gambian  
249 children who did or did not carry *P. falciparum* parasites during the dry season found no difference in  
250 the rate of decline of IgG specific for three *P. falciparum* merozoite antigens (AMA1, EBA175,  
251 MSP1<sub>19</sub>), whereas IgG specific for the merozoite antigen MSP2 declined more rapidly in uninfected  
252 children [37], suggesting interactions between specific antigens and infection status that require  
253 further investigation. We cannot exclude a role for chronic *P. falciparum* infection in maintaining  
254 other facets of host immunity such as cell-mediated immunity or regulatory mechanisms that attenuate  
255 malaria-induced inflammation. Our prior work in Mali suggests that asymptomatic infection during  
256 the dry season maintains *P. falciparum*-inducible IL-10 production capacity in some individuals;  
257 however, the magnitude of this response is much lower than that observed in the same children during  
258 the preceding transmission season one week after acute febrile malaria [38]. This is consistent with  
259 other studies in this population that showed a marked increase in *P. falciparum*-specific memory B

260 cells and antibodies during acute malaria that waned rapidly during the subsequent dry season [39,  
261 40]. Together these observations suggest that the maintenance of malaria immunity depends on  
262 repeated exposures to newly transmitted parasites.

263  
264 Of note, we observed no difference in baseline Hb levels among uninfected subjects and  
265 asymptomatically infected subjects, possibly explained in part by the exclusion of subjects with Hb <7  
266 g/dL from this study. Moreover, treatment of asymptomatic *P. falciparum* infection did not change the  
267 prevalence of anemia one year later (Supplementary Figure 2), which is consistent with a study in  
268 Kenya [41], but at odds with other studies [24, 42].

269  
270 This study has limitations. First, subjects were not blinded to treatment status, which could have led to  
271 differences in treatment seeking behavior. However, this was likely mitigated by weekly active  
272 surveillance for symptomatic malaria. Second, subjects were not randomized to treatment or no  
273 treatment groups, but were classified as such based on the RDT result at the end of the dry season,  
274 which may have led to differences between groups in known and unknown factors that affect malaria  
275 risk. The most important factors known to influence malaria risk in this cohort are age and Hb type  
276 [43], which did not differ significantly between the RDT<sup>+</sup> treated and RDT<sup>-</sup>PCR<sup>+</sup> untreated groups.  
277 Moreover, subjects who were blood smear<sup>+</sup> or blood smear<sup>-</sup>PCR<sup>+</sup> at the end of the first dry season had  
278 the same risk of clinical malaria during the first year of the study. Although we did not control for  
279 socioeconomic factors, the study population was an age-stratified random sample of individuals  
280 residing in a rural community where socioeconomic conditions are relatively uniform and where the  
281 research clinic was the only local source of antimalarials. Moreover, it seems unlikely that  
282 socioeconomic factors confounded the results such that children who were infected with *P. falciparum*  
283 before the malaria season were more likely to experience malaria during the transmission season, since  
284 we observed the opposite effect in this study. Thirdly, a larger study may have detected smaller  
285 differences in the risk of clinical malaria between groups. Finally, the average age of the RDT<sup>+</sup> treated  
286 and RDT<sup>-</sup>PCR<sup>+</sup> untreated groups was ~8 years, so further studies are needed to determine the impact  
287 of treating chronic asymptomatic *P. falciparum* infection in younger children.

288  
289 Recent studies in endemic areas have shown that more sensitive detection methods reveal larger  
290 reservoirs of asymptomatic *P. falciparum* infection than previously appreciated [44, 45]. Therefore, it  
291 is possible that some subjects in this study had parasite densities below the detection limit of our PCR  
292 assay. However, the primary objective of this study was to compare febrile malaria risk in RDT<sup>+</sup>  
293 treated subjects vs. RDT<sup>+</sup>PCR<sup>+</sup> untreated subjects, so the possibility that some PCR<sup>-</sup> subjects were  
294 infected is unrelated to the major conclusions of this study. Moreover, if a significant proportion of  
295 PCR<sup>-</sup> subjects were actually infected, it would be difficult to reconcile their superior ability to  
296 suppress parasitemia during the dry season with their lower breadth and magnitude of *P. falciparum*-  
297 specific antibodies and higher risk of febrile malaria during the ensuing malaria season.

298  
299 Because this study does not support a causal link between chronic asymptomatic *P. falciparum*  
300 infection and protection from febrile malaria, the question remains: what underlies the association  
301 between asymptomatic infection and decreased malaria risk? Longitudinal analysis of *P. falciparum*-  
302 specific IgG responses in this study suggest that asymptomatic infection during the dry season is  
303 simply a marker of higher past *P. falciparum* exposure and thus higher cumulative humoral immunity  
304 to malaria. It is also possible that the protective immunomodulatory effects of asymptomatic infection  
305 persist beyond antimalarial treatment at the end of the dry season into the subsequent transmission  
306 season—a possibility that could be tested by treating asymptomatic infections at the beginning of the  
307 dry season and ensuring that clearance is sustained by repeated screening and MDA.

308  
309 In summary, treatment of chronic asymptomatic *P. falciparum* infection at the end of the dry season  
310 did not change the subsequent risk of clinical malaria, and *P. falciparum*-specific antibodies declined  
311 similarly in children who did or did not harbour chronic asymptomatic *P. falciparum* infection during  
312 the dry season. These findings challenge the notion that chronic asymptomatic *P. falciparum* infection  
313 maintains malaria immunity and suggest that MDA during the dry season may not increase the  
314 subsequent risk of clinical malaria at the individual level.

315

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319

320 **Conflicts of interest statement**

321 The authors of this work have no conflicts of interest.

322

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326

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329 **References**

330

- 331 1. WHO, *World Malaria Report 2015*. 2015.
- 332 2. Bhatt, S., et al., *The effect of malaria control on Plasmodium falciparum in Africa between*  
333 *2000 and 2015*. Nature, 2015. **526**(7572): p. 207-11.
- 334 3. Greenwood, B.M., *Asymptomatic malaria infections--do they matter?* Parasitol Today,  
335 1987. **3**(7): p. 206-14.
- 336 4. Bousema, T., et al., *Asymptomatic malaria infections: detectability, transmissibility and*  
337 *public health relevance*. Nat Rev Microbiol, 2014. **12**(12): p. 833-40.
- 338 5. Ashley, E.A. and N.J. White, *The duration of Plasmodium falciparum infections*. Malar J,  
339 2014. **13**: p. 500.
- 340 6. Bousema, T., et al., *Mosquito feeding assays to determine the infectiousness of naturally*  
341 *infected Plasmodium falciparum gametocyte carriers*. PLoS One, 2012. **7**(8): p. e42821.
- 342 7. Churcher, T.S., et al., *Predicting mosquito infection from Plasmodium falciparum*  
343 *gametocyte density and estimating the reservoir of infection*. Elife, 2013. **2**: p. e00626.
- 344 8. Drakeley, C., et al., *The epidemiology of Plasmodium falciparum gametocytes: weapons of*  
345 *mass dispersion*. Trends Parasitol, 2006. **22**(9): p. 424-30.
- 346 9. Ouedraogo, A.L., et al., *Substantial contribution of submicroscopical Plasmodium*  
347 *falciparum gametocyte carriage to the infectious reservoir in an area of seasonal transmission*.  
348 PLoS One, 2009. **4**(12): p. e8410.
- 349 10. Newby, G., et al., *Review of mass drug administration for malaria and its operational*  
350 *challenges*. Am J Trop Med Hyg, 2015. **93**(1): p. 125-34.
- 351 11. Okell, L.C., et al., *The potential contribution of mass treatment to the control of*  
352 *Plasmodium falciparum malaria*. PLoS One, 2011. **6**(5): p. e20179.
- 353 12. Sturrock, H.J., et al., *Targeting asymptomatic malaria infections: active surveillance in*  
354 *control and elimination*. PLoS Med, 2013. **10**(6): p. e1001467.

- 355 13. Kern, S.E., et al., *Community screening and treatment of asymptomatic carriers of*  
356 *Plasmodium falciparum with artemether-lumefantrine to reduce malaria disease burden: a*  
357 *modelling and simulation analysis*. Malar J, 2011. **10**: p. 210.
- 358 14. Bousema, J.T., et al., *Plasmodium falciparum gametocyte carriage in asymptomatic*  
359 *children in western Kenya*. Malar J, 2004. **3**: p. 18.
- 360 15. Smith, T., et al., *Premunition in Plasmodium falciparum infection: insights from the*  
361 *epidemiology of multiple infections*. Trans R Soc Trop Med Hyg, 1999. **93 Suppl 1**: p. 59-64.
- 362 16. Baird, J.K., *Host age as a determinant of naturally acquired immunity to Plasmodium*  
363 *falciparum*. Parasitol Today, 1995. **11**(3): p. 105-11.
- 364 17. Sergent, E., *[Definition of immunity & premunition]*. Ann Inst Pasteur (Paris), 1950. **79**(5):  
365 p. 786-97.
- 366 18. Bereczky, S., et al., *Multiclonal asymptomatic Plasmodium falciparum infections predict a*  
367 *reduced risk of malaria disease in a Tanzanian population*. Microbes Infect, 2007. **9**(1): p. 103-10.
- 368 19. Crompton, P.D., et al., *Sickle Cell Trait Is Associated with a Delayed Onset of Malaria:*  
369 *Implications for Time-to-Event Analysis in Clinical Studies of Malaria*. J Infect Dis, 2008. **198**(9): p.  
370 1265-1275.
- 371 20. Doumbo, S., et al., *Co-infection of long-term carriers of Plasmodium falciparum with*  
372 *Schistosoma haematobium enhances protection from febrile malaria: a prospective cohort study in*  
373 *Mali*. PLoS Negl Trop Dis, 2014. **8**(9): p. e3154.
- 374 21. Males, S., O. Gaye, and A. Garcia, *Long-term asymptomatic carriage of Plasmodium*  
375 *falciparum protects from malaria attacks: a prospective study among Senegalese children*. Clin  
376 Infect Dis, 2008. **46**(4): p. 516-22.
- 377 22. Sonden, K., et al., *Asymptomatic Multiclonal Plasmodium falciparum Infections Carried*  
378 *Through the Dry Season Predict Protection Against Subsequent Clinical Malaria*. J Infect Dis, 2015.  
379 **212**(4): p. 608-16.
- 380 23. von Seidlein, L., et al., *The effect of mass administration of sulfadoxine-pyrimethamine*  
381 *combined with artesunate on malaria incidence: a double-blind, community-randomized, placebo-*  
382 *controlled trial in The Gambia*. Trans R Soc Trop Med Hyg, 2003. **97**(2): p. 217-25.



- 383 24. Tiono, A.B., et al., *A controlled, parallel, cluster-randomized trial of community-wide*  
384 *screening and treatment of asymptomatic carriers of Plasmodium falciparum in Burkina Faso.*  
385 *Malar J*, 2013. **12**: p. 79.
- 386 25. Larsen, D.A., et al., *Population-wide malaria testing and treatment with rapid diagnostic*  
387 *tests and artemether-lumefantrine in southern Zambia: a community randomized step-wedge*  
388 *control trial design.* *Am J Trop Med Hyg*, 2015. **92**(5): p. 913-21.
- 389 26. Laishram, D.D., et al., *The complexities of malaria disease manifestations with a focus on*  
390 *asymptomatic malaria.* *Malar J*, 2012. **11**: p. 29.
- 391 27. Tran, T.M., et al., *An intensive longitudinal cohort study of Malian children and adults*  
392 *reveals no evidence of acquired immunity to Plasmodium falciparum infection.* *Clin Infect Dis*,  
393 2013. **57**(1): p. 40-7.
- 394 28. Bell, D., C. Wongsrichanalai, and J.W. Barnwell, *Ensuring quality and access for malaria*  
395 *diagnosis: how can it be achieved?* *Nat Rev Microbiol*, 2006. **4**(9 Suppl): p. S7-20.
- 396 29. Bharti, P.K., et al., *The usefulness of a new rapid diagnostic test, the First Response Malaria*  
397 *Combo (pLDH/HRP2) card test, for malaria diagnosis in the forested belt of central India.* *Malar J*,  
398 2008. **7**: p. 126.
- 399 30. Nsoby, S.L., et al., *Molecular evaluation of the natural history of asymptomatic*  
400 *parasitemia in Ugandan children.* *J Infect Dis*, 2004. **189**(12): p. 2220-6.
- 401 31. Anderson, T.J., et al., *Twelve microsatellite markers for characterization of Plasmodium*  
402 *falciparum from finger-prick blood samples.* *Parasitology*, 1999. **119 ( Pt 2)**: p. 113-25.
- 403 32. Doolan, D.L., C. Dobano, and J.K. Baird, *Acquired immunity to malaria.* *Clin Microbiol Rev*,  
404 2009. **22**(1): p. 13-36, Table of Contents.
- 405 33. Marsh, K. and S. Kinyanjui, *Immune effector mechanisms in malaria.* *Parasite Immunol*,  
406 2006. **28**(1-2): p. 51-60.
- 407 34. Greenwood, B., *Review: Intermittent preventive treatment--a new approach to the*  
408 *prevention of malaria in children in areas with seasonal malaria transmission.* *Trop Med Int*  
409 *Health*, 2006. **11**(7): p. 983-91.

- 410 35. Wilson, A.L. and I.P. Taskforce, *A systematic review and meta-analysis of the efficacy and*  
411 *safety of intermittent preventive treatment of malaria in children (IPTc)*. PLoS One, 2011. **6**(2): p.  
412 e16976.
- 413 36. Greenwood, B.M., et al., *Mortality and morbidity from malaria after stopping malaria*  
414 *chemoprophylaxis*. Trans R Soc Trop Med Hyg, 1995. **89**(6): p. 629-33.
- 415 37. Akpogheneta, O.J., et al., *Duration of naturally acquired antibody responses to blood-stage*  
416 *Plasmodium falciparum is age dependent and antigen specific*. Infect Immun, 2008. **76**(4): p.  
417 1748-55.
- 418 38. Portugal, S., et al., *Exposure-dependent control of malaria-induced inflammation in*  
419 *children*. PLoS Pathog, 2014. **10**(4): p. e1004079.
- 420 39. Crompton, P.D., et al., *A prospective analysis of the Ab response to Plasmodium falciparum*  
421 *before and after a malaria season by protein microarray*. Proc Natl Acad Sci U S A, 2010. **107**(15):  
422 p. 6958-63.
- 423 40. Weiss, G.E., et al., *The Plasmodium falciparum-specific human memory B cell compartment*  
424 *expands gradually with repeated malaria infections*. PLoS Pathog, 2010. **6**(5): p. e1000912.
- 425 41. Halliday, K.E., et al., *Impact of intermittent screening and treatment for malaria among*  
426 *school children in Kenya: a cluster randomised trial*. PLoS Med, 2014. **11**(1): p. e1001594.
- 427 42. Tiono, A.B., et al., *Treatment of Asymptomatic Carriers of Plasmodium falciparum with*  
428 *Artemether-Lumefantrine: Impact on the Prevalence of Anemia*. Infect Dis Ther, 2013. **2**(1): p. 47-  
429 58.
- 430 43. Tran, T.M., et al., *Naturally acquired antibodies specific for Plasmodium falciparum*  
431 *reticulocyte-binding protein homologue 5 inhibit parasite growth and predict protection from*  
432 *malaria*. J Infect Dis, 2014. **209**(5): p. 789-98.
- 433 44. Hofmann, N., et al., *Ultra-sensitive detection of Plasmodium falciparum by amplification of*  
434 *multi-copy subtelomeric targets*. PLoS Med, 2015. **12**(3): p. e1001788.
- 435 45. Parr, J.B., et al., *Estimation of Plasmodium falciparum Transmission Intensity in Lilongwe,*  
436 *Malawi, by Microscopy, Rapid Diagnostic Testing, and Nucleic Acid Detection*. Am J Trop Med Hyg,  
437 2016. **95**(2): p. 373-7.

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441 **Table 1. Characteristics of study participants stratified by infection status at the end of**  
 442 **the second dry season.**

parameter	RDT-PCR- (N=434)	RDT-PCR+ (N=55)	RDT+ (N=104)	RDT-PCR+ vs RDT+
% of total	73.20%	9.30%	17.50%	
mean age (yrs) (CI 95%)	5.64 (5.3, 5.9)	8.53 (8.0, 9.0)	8.23 (7.9, 8.5)	ns
% female	48.85%	52.73%	41.75%	ns
mean weight (Kg) (CI 95%)	19.61 (19.0, 20.3)	25.93 (24.6, 27.3)	24.44 (23.5, 25.4)	ns
% Hb As	10.83%	10.91%	4.81%	ns
mean Hb (g/dl) (CI 95%)	11.99 (11.9, 12.1)	12.17 (11.9, 12.4)	12.03 (11.8, 12.2)	ns

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444

445 **Figure Legends**

446 **Figure 1. Asymptomatic *P. falciparum* infection at the end of the dry season**  
447 **independently predicts decreased febrile malaria risk during the ensuing malaria**  
448 **season. (A)** Frequency of clinical malaria episodes every 2 days over three years in a cohort  
449 of 695 subjects aged 3 mos - 25 yrs. Clinical malaria defined as axillary temperature  $\geq 37.5^{\circ}\text{C}$ ,  
450  $\geq 2500$  asexual parasites/ $\mu\text{L}$  of blood and no other cause of fever discernible on physical  
451 exam. **(B)** Age-stratified point prevalence of asymptomatic *P. falciparum* infection detected  
452 by PCR or blood smear at the end of the dry season in May 2011. **(C)** Kaplan-Meier analysis  
453 of time to first febrile malaria episode during the 2011 malaria season stratified by *P.*  
454 *falciparum* infection status in May 2011. Pairwise comparisons by log rank test: PCR<sup>+</sup>smear<sup>+</sup>  
455 vs. PCR<sup>-</sup>smear<sup>-</sup> ( $p < 0.0001$ ); PCR<sup>+</sup>smear<sup>-</sup> vs. PCR<sup>-</sup>smear<sup>-</sup> ( $p < 0.0001$ ); PCR<sup>+</sup>smear<sup>+</sup> vs.  
456 PCR<sup>+</sup>smear<sup>-</sup> ( $p = 0.51$ ). **(D)** Cox model showing the effect of *P. falciparum* infection status in  
457 May 2011 on the risk of febrile malaria during the ensuing 2011 malaria season, adjusted for  
458 covariates. Hazard ratios and 95% confidence intervals are represented by open circles and  
459 horizontal bars, respectively.

460

461 **Figure 2. Genetic evidence that *P. falciparum* infections persist throughout the six-**  
462 **month dry season. (A)** Six *P. falciparum* microsatellite loci were examined in peripheral  
463 blood samples collected from 91 *P. falciparum*-infected subjects in January and May 2012.  
464 Each color represents different allele sizes after adjustment to 3 bp bins. **(B)** Proportion of  
465 subjects with different number of *P. falciparum* clones determined by size differences in the  
466 polymorphic region of *msp2* in January ( $n = 124$ ) and May 2012 ( $n = 128$ ) and overlapping at  
467 the two cross-sectional timepoints ( $n = 90$ ) in the respective age groups.

468 **Figure 3. Treatment of chronic asymptomatic *P. falciparum* infection does not change**  
469 **the subsequent risk of febrile malaria.** (A) Kaplan-Meier analysis of time to first febrile  
470 malaria episode during the 2012 malaria season stratified by *P. falciparum* infection and  
471 treatment status at the end of the dry season in May 2012. Pairwise comparisons by log rank  
472 test: RDT<sup>+</sup>/treated vs. PCR<sup>-</sup> (p<0.0001); RDT<sup>-</sup>PCR<sup>+</sup>/untreated vs. PCR<sup>-</sup> (p<0.0001);  
473 RDT<sup>+</sup>/treated vs. RDT<sup>-</sup>PCR<sup>+</sup>/untreated (p=0.26). (B) Cox model showing the effect of *P.*  
474 *falciparum* infection and treatment status in May 2012 on the risk of febrile malaria during  
475 the ensuing 2012 malaria season, adjusted for covariates. (C) Kaplan-Meier analysis of time  
476 to first febrile malaria episode during the 2013 malaria season stratified by *P. falciparum*  
477 infection and treatment status at the end of the dry season in May 2012. Pairwise comparisons  
478 by log rank test: RDT<sup>+</sup>/treated vs. PCR<sup>-</sup> (p<0.0001); RDT<sup>-</sup>PCR<sup>+</sup>/untreated vs. PCR<sup>-</sup>  
479 (p<0.0001); RDT<sup>+</sup>/treated vs. RDT<sup>-</sup>PCR<sup>+</sup>/untreated (p=0.066). (D) Cox model showing the  
480 effect of *P. falciparum* infection and treatment status in May 2012 on the risk of febrile  
481 malaria during the 2013 malaria season, adjusted for covariates. Hazard ratios and 95%  
482 confidence intervals are represented by open circles and horizontal bars, respectively.

483  
484 **Figure 4. *P. falciparum*-specific IgG reactivity decreases during the dry season**  
485 **irrespective of *P. falciparum* infection status.** (A) Breadth of IgG response in January and  
486 May 2012 stratified by *P. falciparum* infection status in May 2012. (B) Magnitude of IgG  
487 reactivity in January and May 2012 stratified by *P. falciparum* infection status in May 2012.  
488 (C) Magnitude of IgG response in January and May 2012 for antigens that were reactive at  
489 both timepoints (2 SDs above the no DNA control), stratified by *P. falciparum* infection  
490 status in May 2012. (D) Proportion of antigens to which the level of IgG reactivity fell below  
491 the level of detection between January and May 2012 stratified by *P. falciparum* infection  
492 status in May 2012. (E) Change in magnitude of IgG reactivity for antigens that were reactive

493 in January and May 2012, stratified by *P. falciparum* infection status in May 2012. Breadth is  
494 defined as the number of antigens to which the level of IgG reactivity exceeds 2 SDs above  
495 the no DNA control. Magnitude is defined as the sum of  $\log_2$ -IgG intensity values for all  
496 antigens per sample. Boxes indicate median, 25<sup>th</sup> and 75<sup>th</sup> percentiles. Values greater than 1.5  
497 times the IQR are plotted as individual points (Tukey's method).  
498