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

Silvia Portugal[1], Tuan M. Tran[1,2], Aissata Ongoiba[3], Aboudramane Bathily[3], Shanping Li[1], Safiatou Doumbo[3], Jeff Skinner[1], Didier Doumtabe[3], Younoussou Kone[3], Jules Sangala[3], Aarti Jain[4], D. Huw Davies[4], Christopher Hung[4], Li Liang[4], Stacy Ricklefs[5], Manijeh Vafa Homann[6], Philip L. Felgner[4], Stephen F. Porcella[5], Anna Färnert[6,7], Ogobara K. Doumbo[3], Kassoum Kayentao[3], Brian M. Greenwood[8], Boubacar Traore[3], Peter D. Crompton[1]

[1]Laboratory of Immunogenetics, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Rockville, Maryland, USA

[2]Division of Infectious Diseases, Department of Medicine, Indianapolis University School of Medicine, Indianapolis, IN

[3]Malaria Research and Training Centre, Department of Epidemiology of Parasitic Diseases, International Center of Excellence in Research, University of Sciences, Technique and Technology of Bamako, Bamako, Mali

[4]University of California Irvine, Irvine, CA, USA

[5]Rocky Mountain Laboratory Research Technologies Section, Genomics Unit, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Hamilton, Montana, USA

[6]Department of Infectious Diseases Unit Department of Medicine Solna, Karolinska Institutet, Stockholm, Sweden

[7]Department of Infectious Diseases, Karolinska University Hospital, Stockholm, Sweden

[8]Faculty of Infectious and Tropical Diseases, London School of Hygiene & Tropical Medicine, London, UK

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Correspondence to: Dr Silvia Portugal, Laboratory of Immunogenetics, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Twinbrook II, Rm 125, 12441 Parklawn Drive Rockville, MD 20852, USA
silvia.portugal@med.uni-heidelberg.de

Dr Peter D. Crompton, Laboratory of Immunogenetics, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Twinbrook II, Rm 125, 12441 Parklawn Drive Rockville, MD 20852, USA
pcrompton@niaid.nih.gov

Key Points:

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

Abstract

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

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

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

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

Introduction

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

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

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

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

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

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

METHODS

Ethics statement

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

Study design and participants

From May 2011 through December 2013 a cohort study was conducted in Kalifabougou, Mali, a rural village of ~5000 inhabitants where malaria transmission occurs from July through December. A single clinic and pharmacy provided the only access to antimalarial drugs. A detailed description of the study site and cohort design has been published elsewhere [27]. From an age-stratified, random sample of the entire village population, 695 healthy individuals aged six months to 25 years were enrolled. Exclusion criteria were haemoglobin concentration <7 g/dL, axillary temperature ≥37.5°C, acute systemic illness, or use of antimalarial or immunosuppressive medications in the preceding 30 days. Clinical malaria episodes were detected prospectively by active and passive surveillance and were defined by an axillary temperature of ≥37.5°C, ≥2500 asexual parasites/μL of blood, and no other cause of fever on physical examination.

Detection of *P. falciparum* infection

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

Additional methods are described online in supplementary information.

Results

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

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

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

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

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

the second dry season (May 2012) using an RDT with a sensitivity comparable to blood smear [29]. All subjects found to be *P. falciparum*-infected by RDT (n=104) were treated with a standard 3-day course of AL, the first daily dose of which was directly observed by study staff. Dried blood spots collected from RDT^- subjects at the same timepoint (n=489) were later analyzed by PCR to retrospectively identify two additional groups that did not receive antimalarials: RDT^-PCR^+ subjects (n=55) and RDT^-PCR^- subjects (n=434).

Because asymptomatic *P. falciparum* infections detected during cross-sectional surveys can become symptomatic within days or weeks of initial detection [3, 4, 30], we sought to confirm that *P. falciparum* infections detected at the end of the dry season in May 2012 were chronic and asymptomatic. We found that subjects infected with *P. falciparum* in May 2012 were highly likely to have been infected at the start of the dry season (January 2012) [OR: 842.6 c.i. (200.2, 3546.3), $P < 0.0001$]; and through the mid-dry season (March 2012) [OR: 172.9 c.i. (79.8, 374.5), $P < 0.0001$]; and conversely, uninfected subjects at the start of the dry season remained uninfected at the end of the dry season (Supplementary Figure 1). During the same time period, no cases of clinical malaria were detected. We also examined parasites collected in January and May 2012, from individuals who tested PCR^+ in May 2012, for 6 microsatellite loci previously used to characterize the genetic diversity of *P. falciparum* [31], and we obtained low F_{st} values between the populations (F_{st} January *vs.* May = 0.004), indicating that the two populations were genetically very similar (Figure 2A). Additionally, analysis of the polymorphic region of the *P. falciparum msp2* locus in January and May 2012 indicated that asymptomatic infections were polyclonal at both timepoints and 47% of subjects harboured at least one common parasite clone at both timepoints (Figure 2B). Together with our observation that the entomological inoculation rate is near zero during the dry season, these data indicate that asymptomatic *P. falciparum* infections detected at the end of the dry season had persisted as chronic asymptomatic infections throughout the preceding dry season.

The characteristics of the three groups defined in May 2012 (RDT^+ treated, RDT^-PCR^+ untreated, and RDT^-PCR^- untreated) are shown in Table 1. Consistent with the first year of the study, the RDT^-PCR^-

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

P. falciparum-specific humoral immunity decreases similarly with or without chronic asymptomatic infection

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

Discussion

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

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

We found that the breadth and magnitude of IgG specific for 862 *P. falciparum* proteins/polypeptides declined at a similar rate in children who did or did not carry asymptomatic *P. falciparum* infection during the dry season. Similarly,Consistent with our antibody data, a study comparing Gambian children who did or did not carry *P. falciparum* parasites during the dry season found no difference in the rate of decline of IgG specific for three *P. falciparum* merozoite antigens (AMA1, EBA175, $MSP1_{19}$), whereas IgG specific for the merozoite antigen MSP2 declined more rapidly in uninfected children [37], suggesting interactions between specific antigens and infection status that require further investigation. We cannot exclude a role for chronic *P. falciparum* infection in maintaining other facets of host immunity such as cell-mediated immunity or regulatory mechanisms that attenuate malaria-induced inflammation. Our prior work in Mali suggests that asymptomatic infection during the dry season maintains *P. falciparum*-inducible IL-10 production capacity in some individuals; however, the magnitude of this response is much lower than that observed in the same children during the preceding transmission season one week after acute febrile malaria [38]. This is consistent with other studies in this population that showed a marked increase in *P. falciparum*-specific memory B

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

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

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

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

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

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

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Conflicts of interest statement

The authors of this work have no conflicts of interest.

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Table 1. Characteristics of study participants stratified by infection status at the end of 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

Figure Legends

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

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

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

Figure 4. *P. falciparum*-specific IgG reactivity decreases during the dry season irrespective of *P. falciparum* infection status. (**A**) Breadth of IgG response in January and May 2012 stratified by *P. falciparum* infection status in May 2012. (**B**) Magnitude of IgG reactivity in January and May 2012 stratified by *P. falciparum* infection status in May 2012. (**C**) Magnitude of IgG response in January and May 2012 for antigens that were reactive at both timepoints (2 SDs above the no DNA control), stratified by *P. falciparum* infection status in May 2012. (**D**) Proportion of antigens to which the level of IgG reactivity fell below the level of detection between January and May 2012 stratified by *P. falciparum* infection 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^{th} and 75^{th} percentiles. Values greater than 1.5
497 times the IQR are plotted as individual points (Tukey's method).
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