

## USE OF AREA UNDER THE CURVE TO CHARACTERIZE TRANSMISSION POTENTIAL AFTER ANTIMALARIAL TREATMENT

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**Abstract.** To evaluate transmission potential of *Plasmodium falciparum*, we use the area under the curve (AUC) of gametocyte levels after treatment as an approach to combine their duration and magnitude. Analysis of determinants of AUC was based on two main exposures: parasite clearance time (PCT) and presence of dihydrofolate reductase and dihydropteroate synthase mutations associated with sulfadoxine-pyrimethamine (SP) resistance *in vitro*. Exposures were determined based on the first three days after treatment with SP of 96 individuals who had malaria, cleared parasitemia by days 1–3, and were followed-up for 21 days. Using regression methods, we characterized both the heterogeneity of the presence of gametocytes (AUC > 0) and the magnitude of the AUC among those with an AUC > 0. A PCT of two or three days was associated with a substantial and highly significant odds ratio for presence of gametocytes. Among those who developed gametocytes, if their clearance time was 3 days or if they had any mutations (1 or 2) the magnitude of gametocytemia was  $\geq 3$ -fold. Methods presented are applicable to both observational studies and clinical trials assessing the effect of therapies on transmission potential.

### INTRODUCTION

Drug-resistant *Plasmodium falciparum* is a major threat to global public health and new strategies are needed to deter spread of resistance to available and forthcoming antimalarial drugs. Resistance is evaluated *in vivo* by measuring asexual parasitologic response after treatment, and measures of antimalarial drug treatment efficacy incorporate both clinical and parasitologic responses to treatment.<sup>1</sup> However, particularly where drug resistance is emerging, clearance of gametocytes (i.e., the sexual forms of the parasite that are infectious to mosquitoes) is also a goal of malaria treatment, not only because of the need to reduce transmission but also to prevent the selection and propagation of resistant parasites. Therefore, when evaluating the efficacy of antimalarial treatments, gametocytemia after treatment has become an outcome of interest because it provides an estimate of the transmission potential of resistant parasites.

Development of gametocytemia is evaluated by serial microscopic examination of thick blood smears after drug administration. Gametocytemia is usually measured on the day of treatment and 7, 14, 21, and 28 days after treatment, during which it usually peaks and then starts to decrease. Studies frequently evaluate the impact of drugs on gametocytemia after treatment by measuring the gametocyte carriage rate on these days.<sup>2–6</sup> Typically, two descriptors of the gametocyte distribution have been used: 1) the proportion of individuals with detectable gametocytes at a particular day after treatment (percentage > 0 or discrete component), and 2) mean gametocytemias, excluding those with no gametocytes (mean for those that are > 0 or continuous component).

We propose the use of an alternative outcome for transmission potential that can incorporate magnitude and duration of gametocytemia: the area under the curve (AUC) of the gametocyte levels after treatment. The main advantage of our approach is that the outcome incorporates the longitudinal data of the gametocytes levels collected at several days.

We examined this analytic approach using data from a study designed to evaluate determinants of treatment response to sulfadoxine-pyrimethamine (SP) among *P. falciparum*-infected patients in the Pacific coast region of Colombia.

In this region, resistance to SP has remained low in contrast to high rates of SP failure elsewhere in South America. However, we have reported that known parasite mutations that confer low-level resistance to SP, even though insufficient to cause treatment failure, may contribute to the potential for the transmission of *P. falciparum* and the spread of resistance.<sup>5</sup> Specifically, point mutations in parasite dihydrofolate reductase (DHFR) that confer *in vitro* resistance to pyrimethamine were associated with longer parasite clearance time (PCT) and the presence of gametocytes after SP treatment.<sup>5</sup> This finding suggested that even before clinical SP resistance is apparent drug treatment may be responsible, by increased gametocytemia, for selecting resistant parasites and contributing to the spread of resistance.

This report evaluates the use of the AUC of the gametocyte distribution after treatment as an alternative way to measure transmission potential. We describe methods for characterizing the joint effects of PCT and DHFR mutations at codons 108 and 51 on two outcomes: presence of gametocytes and magnitude of gametocytemia among treated individuals in whom gametocytes are present. We characterized the role of the explanatory variables (i.e., PCT and mutations) on the two types of outcomes, and this characterization can be helpful in assessing the ability of antimalarial treatment regimens to prevent the occurrence of gametocytes and/or to reduce the magnitude of gametocytemia.

### MATERIALS AND METHODS

**Study area.** The study was carried out in Buenaventura, Colombia, a seaport located in a highly humid tropical forest zone on the Pacific Coast of Colombia. The mean temperature is approximately 28°C and annual rainfall ranges from 6,000 to 9,000 mm. The municipality has approximately 300,000 inhabitants, with 85% living in the urban area. Malaria is hypoendemic and its incidence has a long-term periodicity with peaks occurring every four years. Fifty to 80% of malaria is caused by *P. falciparum*, which is usually more

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frequent than *P. vivax* malaria during epidemic years. The annual incidence is approximately 60–100/1,000 in the rural area and 1–3/1,000 in the urban area. Malaria occurs throughout the year, with two seasonal peaks usually between April and May and between September and October.<sup>7</sup>

**Study population.** One hundred twenty subjects with acute *P. falciparum* malaria were enrolled in the study, received standard SP treatment, and were followed-up for 21 days or until treatment failure. The study protocol was reviewed and approved by the Universidad del Valle Ethics Committee in compliance with national regulations governing the protection of human subjects. All subjects provided written informed consent. Subjects were 1–70 years old (median = 21.2 years), 63% were male, and 88% lived in the urban area of the study. Four individuals had treatment failure, received rescue treatment, and were not included in the analysis of the distribution of gametocytes. In addition, parasite DNA from blood samples of 20 individuals could not be amplified by polymerase chain reaction, which prevented the assessment of mutations for DHFR and dihydropteroate synthase (DHPS). The 96 individuals with available data on presence of DHFR mutations and with parasites clearance time on days 1, 2, and 3 comprised the study population.

**Outcome variable: AUC of gametocytemia after treatment.** Gametocyte density was determined on days 0, 3, 7, 14, and 21 after treatment from thick blood films by counting the number of sexual parasites per 200 white blood cells (WBCs) (or per 500 if the count was less than 10 parasites/200 WBCs) and calculating parasites per microliter assuming a WBC count of 8,000/ $\mu$ L.

The AUC is a summary calculation used when serial measurements on each subject under study are carried out. Alternative outcomes include mean of all measurements, height of peak, time to reach peak and time to return to baseline level.<sup>8</sup> The AUC is frequently used in clinical pharmacology, where estimates of the area made from drug serum levels over time can be interpreted as the total uptake of the drug. In the case of assessing the response to antimalarial treatment, we propose using AUC of the gametocyte distribution after treatment because it incorporates both the magnitude and the duration of transmission potential.

The AUC from days 3 to 21 was calculated as  $AUC = [(7 - 3) \times (g_3 + g_7)/2 + (14 - 7) \times (g_7 + g_{14})/2 + (21 - 14) \times (g_{14} + g_{21})/2]/(21 - 3)$  where  $g_d$  represents gametocyte density on day  $d$ . We scaled this area by 18 (21 - 3) so that it represents AUC per day and transformed this by  $\log_{10}$  to make its distribution to be amenable to methods based on the Gaussian bell shaped distribution. Specifically, the outcomes used in all analyses that follow were 1) a binary outcome indicating presence of gametocytes, and 2) the  $\log_{10}$  (AUC) if  $AUC > 0$ .

The four values of gametocytes at days 3, 7, 14, and 21 were required to have the AUC as a complete outcome measure. For instances when a value was missing between two available values (e.g., missing on day 14 with values available on days 7 and 21), simple interpolation (i.e., mean) of the available data was used to complete the missing data. The other type of missing data was that of not having data on any subsequent days. Fifteen (16%) of the 96 individuals had missing data on day 21 and only 3 (3%) had missing data on days 14 and 21. We completed the data for each of these 18 individuals by using the observed data in individuals with similar values of gametocytemia at all the visits if the 18 individuals have data

available. Thus, we used the standard and simple “hot deck” method for completing missing data.

To estimate the effect of these imputations we repeated the analysis using only observed data with heterogeneous total length of follow-up. In particular, for the 18 individuals with missing data the AUC corresponds to the previous formula until days 7 or 14.

**Determinants of AUC.** Analysis of determinants of AUC was based on two main exposures: PCT and presence of mutations *in vitro* associated with SP resistance at DHFR and DHPS codons. Polymerase chain reaction methods to assess parasite mutations were applied according to protocols described elsewhere<sup>9</sup> and available from <http://medschool.umaryland.edu/CVD/plowe.html>.

Since we restricted our study population to the individuals who cleared parasites on days 1, 2, or 3, this exposure preceded the AUC from days 3 to 21, which was used as the outcome variable. The PCT (1, 2, or 3 days) and mutations (none, one [108], or 2 [108 and 51]) were treated as indicator variables in all regression models using no mutation and PCT = 1 as the reference categories.

**Statistical analysis.** Given that the number of subjects who maintain 0 gametocytes over the post-treatment period (i.e.,  $AUC = 0$ ) was not uncommon, the true distribution of AUC had a mass at 0 with probability  $1 - \pi$  and we modeled the  $\pi$  values greater than 0 as a normal distribution (in the log scale). To characterize differences in AUC according to exposures, we performed analysis in two stages. In the first stage, we used the full population and implemented logistic regression methods for the odds of gametocytes being present at any time to assess the predictors of AUC being greater than 0 (i.e., presence of gametocytes). In the second stage, we restricted to those with  $AUC > 0$  and implemented standard linear regression methods for the  $\log_{10}$  (AUC) to describe the heterogeneity of the magnitude of gametocytemia. Both models were run as univariate by either mutations or clearance time and also as multivariate with both mutations and clearance time in the models. For both regressions we used standard methods of maximum likelihood estimation and we estimated the 95% confidence intervals for their corresponding coefficients.

## RESULTS

Table 1 shows cross-sectional descriptive statistics of the gametocytes for the 96 individuals comprising the study population at days 3, 7, 14, and 21. For the 11 instances with missing data on gametocytes in a given day but with available data before and after, we completed the data by simple interpola-

TABLE 1  
Descriptive statistics of gametocytes on days 3, 7, 14 and 21 after SP treatment among patients with uncomplicated *Plasmodium falciparum* malaria, Buenaventura, Colombia, 1999–2000\*

Variable	Day after treatment			
	3	7	14	21
No. with no gametocytes	73	24	23	37
No. with gametocytes	22	72	60	41
No. missing with subsequent data	1	0	10	NA
No. missing with no subsequent data	0	0	3	18

\* SP = sulfadoxine-pyrimethamine; NA = not applicable.

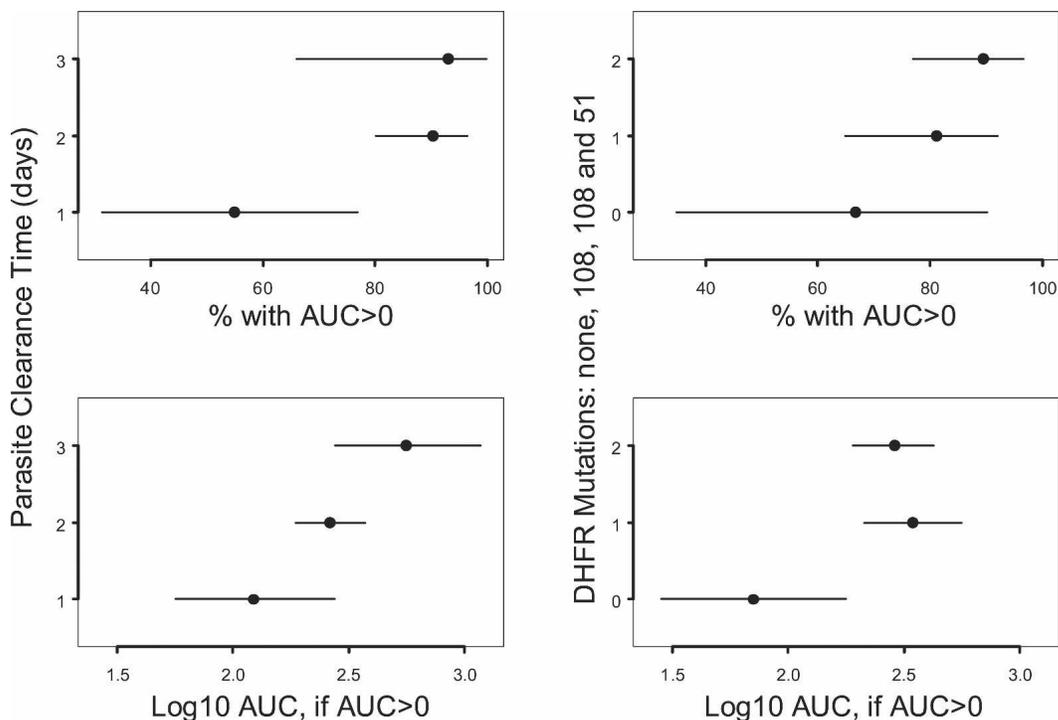


FIGURE 1. Association between parasite clearance time (PCT), dihydrofolate reductase (DHFR) mutations, and area under the curve (AUC) of gametocytemia. ● = point estimates. Horizontal lines represent 95% confidence intervals.

tion. There were three individuals with missing data on days 14 and 21 and 15 (18 – 3) with missing data on day 21. The missing data of these 18 individuals was completed by using the observed data in individuals with similar values of gametocytemia at all the visits if the 18 individuals had data available.

The percent of individuals with AUC > 0 and the mean log<sub>10</sub> of AUC for those with AUC > 0 according to the PCT were 55% and 2.09 for PCT = 1 day, 90% and 2.41 for PCT = 2 days, and 93% and 2.75 for PCT = 3 days. The corresponding values for AUC according to DHFR mutations status were 67% and 1.85 for wild infections, 81% and 2.54 for 108 single-mutant infections, and 89% and 2.46 for 108 and 51 double-mutant infections. Figure 1 shows these descriptive statistics along with the corresponding 95% confidence intervals. For the presence of gametocytes (i.e., % AUC > 0) the odds are increased by prolonged PCT and by number of mutations, but for PCT there is a threshold effect at 2 and a trend according to the number of mutations. In contrast, the magnitude of AUC follows a dose response with longer PCT but has a threshold effect according to absence or presence of mutations.

When only the observed incomplete data were used (i.e., no imputation), no changes were observed in the percent of individuals with AUC > 0 and only small variations were seen in the magnitude of AUC. In particular, mean values were 2.17, 2.50, and 2.86 for PCT = 1, 2, and 3, respectively, and mean AUC were 1.92, 2.62, and 2.55 for wild infections, 108 only mutant infections, and 108/51 double-mutant infections. Consequently, the statistically significant (*P* < 0.05) differences for PCT = 3 days and the single and double mutations were preserved when we restricted the analysis to the observed data (i.e., no imputations).

Table 2 shows the univariate and bivariate analyses of the effects of PCT and mutations on presence of gametocytes. In the bivariate model, although the effect of mutations suggest a dose response, it was not statistically significant. In contrast, PCT showed that the presence of gametocytes was significantly increased by PCT > 1 day, but was not affected by PCT increasing from two to three days.

Table 3 shows the univariate and bivariate analysis of the effects of PCT and mutations on the magnitude of the log<sub>10</sub> AUC of gametocytes values for those on whom AUC was greater than zero. In the bivariate model, interpretation of these coefficients by exponentiation showed a similar and significant 3–4-fold increase in magnitude of AUC with a single mutation (i.e., 4.0 = 10<sup>0.60</sup> for 108 mutation) and a double mutation (i.e., 2.9 = 10<sup>0.46</sup> for 51 and 108 mutations). In contrast, only those with PCT = 3 showed a significant four-fold increase of magnitude of gametocytemia (i.e., 3.7 = 10<sup>0.57</sup>).

TABLE 2

Association analysis of PCT and DHFR mutations with presence of gametocytes\*

	Univariate analysis, OR (95% CI)	Bivariate analysis, OR (95% CI)
PCT, days		
1	1	1
2	7.6 (2.26, 25.83)	7.3 (2.13, 25.33)
3	10.6 (1.16, 97.54)	7.3 (0.72, 73.04)
DHFR mutations		
None	1	1
108	2.1 (0.50, 9.18)	2.0 (0.40, 10.00)
108 and 51	4.2 (0.92, 19.14)	3.3 (0.59, 18.04)

\* PCT = parasite clearance time; DHFR = dihydrofolate reductase; OR = odds ratio, CI = confidence interval.

TABLE 3

Association analysis of PCT and DHFR mutations with magnitude of gametocytemia quantified by  $\log_{10}$  (AUC) for those with AUC > 0\*

	Univariate analysis regression coefficients (95% CI)	Bivariate analysis regression coefficients (95% CI)
PCT, days		
1	2.09	1.71
2	0.32 (-0.05, 0.70)	0.23 (-0.14, 0.60)
3	0.66 (0.19, 1.13)	0.57 (0.09, 1.04)
DHFR mutations		
None	1.85	1.71
108	0.69 (0.24, 1.14)	0.60 (0.16, 1.05)
108 and 51	0.61 (0.18, 1.04)	0.46 (0.01, 0.90)

\* PCT = parasite clearance time; AUC = area under the curve; CI = confidence interval; DHFR = dihydrofolate reductase.

In summary, PCT is the main determinant of presence of gametocytemia, and among those who develop gametocytes, if their clearance time is longer (i.e., PCT = 3 days) or if they have any mutations (1 or 2), the magnitude of gametocytes is three-fold or more.

## DISCUSSION

Elimination of asexual forms of *Plasmodium* parasites causing disease is the main clinical goal of antimalarial treatment at an individual level. However, the ability to block transmission of drug-resistant malaria parasites by arresting development of gametocytes is increasingly recognized as an important public health outcome measure of antimalarial drug treatment.<sup>10</sup> Previously, this has been typically done using a cross-sectional approach to compare gametocyte levels at fixed intervals after treatment. Gametocyte prevalence at days 7–28 and their corresponding geometric means among those gametocytemic are used to compare the impact of antimalarial drugs or to evaluate determinants of transmission potential.<sup>2–6,11–13</sup> Duration of gametocytemia may be evaluated with a cohort approach as the interval between the first and last positive smears for gametocytes (i.e., gametocyte clearance time)<sup>14</sup> or by adding patent gametocytemias among individuals under follow-up to estimate gametocyte person-weeks.<sup>2,15</sup> The main comparative advantage of the methods we present is that succinctly incorporate both duration and magnitude of gametocytemia into a single measure defined by the AUC. Furthermore, by calculating the area starting a day after the assessment of the exposure we preserved the desired ordering of exposures preceding the outcome. In addition, the AUC naturally lends itself to carrying out the analysis in two stages whereby the first stage characterizes the presence of gametocytes and the second the magnitude of gametocytes among those in whom gametocytes are present.

To overcome potential limitations due to missing data, we applied simple methods of imputation of missing data based on the observed data to achieve complete data for each individual. Comparative analysis with available data showed that these imputations had not influence our results.

We have shown how AUC can be used to study the transmission potential of *Plasmodium* after treatment in an observational setting. Clinical trials that compare the efficacy of antimalarial drugs used alone or in combination could incorporate the estimation of AUC and help to define which treat-

ments would potentially be better able to deter the rapid appearance of resistance.

In this study, we showed that a prolonged PCT is the primary explanatory variable of the presence of gametocytes. These results are consistent with a previous report by Price and others<sup>2</sup> that showed a significant trend between longer time to clear parasitemia and gametocyte carriage rate. Our results suggest that a PCT of two or three days is associated with a similar substantial and highly significant odds ratio for presence of gametocytes. This suggests that rapidly acting treatments that clear parasites by the first day after treatment may effectively reduce transmission of resistance. In addition, for magnitude of gametocytemia we showed that both PCT and mutations are important, whereby a PCT prolonged to three days is significantly associated to a high magnitude, and that the presence of any mutations is equally deleterious towards an increase in the magnitude of gametocytemia.

Antimalarial drugs that inhibit gametocytogenesis or kill mature gametocytes have the potential to interrupt transmission of malaria. Given in combination, these therapies have been proposed as strategies to secure a longer life for available drugs.<sup>16</sup> However, a comprehensive evaluation of the efficacy of the different drug combinations requires valid and comprehensive methods of analysis, as those we propose in this report.

A limitation of our study is that we did not assess the actual transmission of gametocytes detected in blood smears but just presence and magnitude. The transmission capacity of gametocytes should be confirmed when possible through direct-feeding assays or membrane-feeding assays. In a study in The Gambia, gametocytes emerging after successful treatment of *P. falciparum*-infected children with chloroquine were significantly more likely to be those with resistance genotypes and were infective to mosquitoes.<sup>10</sup>

Based on the shape of the dose response curve between gametocytemia levels and transmission to mosquitoes, it is expected that at similar conditions of malaria endemicity, higher levels of gametocytemias among treated individuals should increase the source of new infections. In particular, infectivity to mosquitoes was shown to present a log-sigmoid relationship with gametocyte density in original malaria therapy studies,<sup>15</sup> but this may vary considerably according to several factors, including the level of transmission-blocking activity among the population, which is affected by previous malaria episodes<sup>17</sup> and variable infectivity of circulating gametocytes.<sup>18</sup>

The data analysis methods presented provided means to jointly characterize the effect of PCT and mutations on the presence and magnitude of gametocytes over the period of 18 days after the third day after treatment with SP. These methods are applicable to both observational studies and clinical trials assessing the effect of therapies on transmission potential.

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