DETERMINANTS OF PARASITE CLEARANCE TIME AND RECRUDESCENCE IN PATIENTS TREATED FOR UNCOMPLICATED *PLASMODIUM FALCIPARUM* MALARIA IN SUB-SAHARAN AFRICA

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A dissertation submitted to the faculty of the University of North Carolina at Chapel Hill in partial fulfilment of the requirements for the degree of Doctor of Philosophy in the Department of Epidemiology in the Gillings School of Global Public Health.

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ABSTRACT

Mary A. Woessner: Determinants of Parasite Clearance Time and Recrudescence in Patients Treated for Uncomplicated *Plasmodium falciparum* Malaria in Sub-Saharan Africa
(Under the direction of Steven R. Meshnick)

Drug-resistant *P.falciparum* malaria is an increasing concern in sub-Saharan Africa (SSA), where previously effective monotherapy treatments were replaced by artemisinin combination therapies (ACT) because of resistance. ACTs typically have a good safety profile and high efficacy responses (>95%). Resistance to ACTs has been documented in Asia, but has not been observed in SSA. However, there are now signs artemisinin-resistance may be spreading to the African continent.

An indication of artemisinin resistance is delayed parasite clearance time (PCT), defined as a slope half-life >5 hours (time to clear 50% of parasites). Artemisinin resistance may also cause ACT treatment failure (recrudescence). It is not clear though if delayed PCT or recrudescence is consistently explained by *parasite factors* such as resistance or *host factors* such as immunity. Exploring the determinants of both delayed PCT and recrudescence is the aim of this dissertation.

Data from a large multi-center clinical trial conducted in 2006-7 when ACTs were first introduced in SSA were utilized for these analyses (n=1372). Candidate variables included: baseline parasitemia, sex, treatment, and surrogate factors for immunity (age, estimated endemicity, seasonality, previous malaria episodes, and geographic location). Logistic regression was used to assess significant factors associated with each outcome.

In Aim 1, we found 24 cases with delayed PCT. Since the study occurred prior to the widespread introduction of ACTs in Africa, our data suggests that resistant parasite strains pre-existed the introduction of ACTs. Site ($p<0.001$) and baseline parasitemia ($p<0.001$) were significantly associated with delayed PCT.

In Aim 2, slope half-life ($p<0.001$), treatment ($p=0.004$), seasonality ($p=0.008$) and endemicity ($p=0.018$) were significant factors in explaining recrudescence, with age ($p=0.076$) and sex ($p=0.106$) also having marginal contributions.

In summary, our findings suggest that delayed PCT pre-dated the widespread use of artemisinin in Africa and that recrudescence may be related to environmental, host and parasite factors.

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CHAPTER 1: SPECIFIC AIMS

The spread of artemisinin resistance to sub-Saharan Africa (SSA) is of great concern since there currently are no other treatment options available for widespread use for *Plasmodium falciparum* malaria and 90% of the world's malaria deaths occur in Africa - the majority in SSA [113]. It is of interest to understand if there is an increased risk of artemisinin-resistance in malaria patients in SSA. Research has been conducted in Asia where resistance to artemisinins or artemisinin containing therapies (ACTs) has been documented after patients have experienced slower clearance of parasites or failure of the treatment, but this assessment has been very limited in SSA where resistance was not thought to have occurred yet [16, 61, 62]. But recent studies have shown that mutations associated with artemisinin resistance can now be found in SSA [3]. But were they pre-existing or recently selected? Therefore any information on delayed parasite clearance or response failure due to recrudescence (return of the original parasite) pre-dating the widespread use of ACTs in SSA would be useful.

The goal of this research is to investigate if there are any clinical indicators which may explain why some patients experience slowed clearance after treatment and separately to assess why some patients may experience an initial response followed by a return of the initial parasite infestation (defined as recrudescence). In the absence of clinical indicators tied to host factors such as immunity or insufficient treatment, the possibility of artemisinin-resistance may be indicated.

Data collected in clinical practice is not often sufficient to provide this type of investigation. Patients are often not monitored in clinic and assessing timing of clearance would require multiple blood draws which again would not be feasible or likely to have occurred. Also, large sets of data are needed to investigate the several potential candidates as determinants. In

order to explore this, retrospective analyses utilizing study data from a large, multi-center, randomized controlled trial of ACTs, where patients were monitored in-clinic every 8 hours to adequately assess parasite clearance and then followed for 42 days to assess if recurrence of the infection occurred were planned.

The specific aims of these analyses were to:

1) Assess if there are clinical determinants of Parasite Clearance Time (PCT) in patients treated with ACTs for *P.falciparum* malaria in SSA.
2) Assess if there are clinical determinants of Recrudescence in patients treated with ACTs for *P.falciparum* malaria in SSA.

The analyses for this dissertation utilized data previously collected in a well-controlled clinical trial, conducted under good clinical practice (GCP) standards. The ancillary analysis made use of data already available and ensured no additional harm was posed to subjects in the conduct of this research. The results of this study may have important implications for indicating if resistance may be occurring in this region or possibly if resistant parasite strains pre-existed the introduction of ACTs in SSA.

CHAPTER 2: BACKGROUND

1. Malaria burden in Sub-Saharan Africa

The World Health Organization estimates there are 198 million malaria cases annually, causing over half a million deaths and 453,000 deaths in children under 5 years, with 90% of all malaria deaths occurring in Africa. Malaria is the leading cause of death in young children in Africa, where a child dies every minute [113]. Hundreds of millions of African children and adults are chronically infected with malaria. Between 30 and 50 percent of inpatient admissions and half of all outpatient visits are attributed to malaria each year [85]. Beyond the human toll, malaria has significant economic impacts in the endemic countries - costing Africa US$12 billion in lost GDP every year and consuming 40 percent of all public health spending [32, 75].

P. falciparum malaria (the most deadly species of the parasite and the predominant form in Africa) is a common cause of mortality in young children throughout sub-Saharan Africa and leads to significant morbidity across all age groups. However, many of the previous first line monotherapy drugs for treating uncomplicated malaria are compromised by parasite resistance. The World Health Organization (WHO) currently advocates 'artemisinin combination therapies' (ACTs) such as: artemether plus lumefantrine, artesunate plus amodiaquine, artesunate plus mefloquine, and artesunate plus sulfadoxine-pyrimehtamine as first-line therapy to forestall resistance. The selection of a particular treatment for a country or region is based on the level of resistance to the partner drug used in combination with the artemisinin [110]. Any sign of resistance to artemisinin in SSA would be an immediate cause for concern due to the lack of other viable treatment options and the devastating situation that would likely follow [23, 62, 67, 104].

2. Artemisinin Resistance

2.1. History and Indication of Artemisinin Resistance

ACTs are currently the first-line treatment recommended by the WHO for the treatment of uncomplicated *Plasmodium falciparum*. In the past monotherapy treatments offered successful treatment, but one by one became less effective due to the development of resistance [42, 79, 116]. Artemisinins (derived from the leaves of the Artemisia annua shrub from China) were initially used as a Chinese herbal medicine with a long history and used to treat fever for many years. In 1967 it was screened and found to have anti-malarial properties and was purified for use as an anti-malarial treatment in the 1970s with widespread use in China in the 1980s [51]. Artemisinins are well-tolerated, offer a good safety profile, and rapid reduction in parasites [50, 85, 101]. They have fast clearance - so an artemisinin monotherapy treatment is not a preferred treatment option as it would require an extended treatment period to remain active and a longer treatment period could promote non-compliance and also allow an increased risk of developing resistance. Creating a combination of artemisinin with another slow-eliminating anti-malarial monotherapy would offer faster clearance of the parasites, extended protection time and therefore a reduced ability for mutation leading to resistance to occur [50, 100, 101]. Also, testing in the laboratory settings to try to introduce resistance in ACTs showed no stable patterns which could be developed [59, 102]. This initially seemed to provide a comprehensive and effective approach to forestall resistance. However, relying on one key component therapy for *Plasmodium falciparum* elimination world-wide could be risky if resistance were discovered; and the potential for resistant strains spreading across regions and continents would be a real fear [67, 102].

There is currently evidence of artemisinin resistance in Asia and it is feared to be moving to the African continent [40, 84, 95].

2.2. Detection of Artemisinin Resistance

Resistance has been an issue for anti-malarial treatments in the past, especially with monotherapy and even in combination therapies [7, 67, 116]. Multidrug resistance has been documented where a protein mediating the resistance can confer resistance or cross-resistance to multiple even structurally unrelated drugs. This is the case for the *pfmdr1* (*Plasmodium falciparum* multi-drug resistant) transport – which was often detected at some level when resistance occurred in the past [20, 21, 44]. This however has not been detected with the current cases where artemisinin resistance has been suspected [25].

Research is ongoing to identify molecular markers for artemisinin resistance. Mutations have been correlated with delayed parasite clearance time from various in vivo and in vitro studies [2, 13, 37, 53, 56, 57, 64]. However, the most promising marker is the gene encoding the K13 (kelch) propeller domain (PF3D7_1343700), which was identified initially by Ariey in 2014 and subsequently by several other independent studies [2, 3, 13, 40, 56, 68, 95, 97]. The mutations correlating with delayed parasite clearance from patient data are shown below in Table 2.1.

Table 2.1. K-13 Mutations which Correlate with Delayed Parasite Clearance

Mutation	Reference
H719N	2
A675V	2,6
F673I	6
F614L	2
Q613E	2
D584V	4,6
C580Y	1,2,4,5,6
A578S	2
P574L	2,4,6
V568G	2,4,6
R561H	2,6
P553L	2,4,6
I543T	2,3,4,6
R539T	1,2,4,5,6
G538V	2,6
N537I	2
N525D	2
N522C	2
R516Y	5
Y493H	1,2,4,6
A481V	2,4,5,6
M476I	2,5
N458Y	2,5,6
G449A	2,4,6
F446I	2,6
P441L	2,6
K438N	2#
D353Y	6
D281V	2#,6#
R255K	2#,4#,6#
E252Q	2#,6#
R223K	2#
K189T	2,4,6#
A175T	2#,6#
T149S	2#
L143P	2#
E130G	2#,6#
G112E	2#
N87K	2#

Samples from regions in Southeast Asia: Bangladesh, Cambodia, Laos, Myanmar, Vietnam.
1-Ariey, 2014: Delayed PCT defined as slope half life >5 hour.
2-Ashley, 2014: Delayed PCT defined as slope half life >5 hours (# indicates all results with half-life <5 hours).
3-Thriemer, 2014: Delayed PCT defined as Parasite Clearance Time > 72 hours.
4-Takala-Harrison, 2015: Delayed PCT defined as slope half life >5 hours (# indicates half-life <5 hours).
5-Nyunt, 2015: Delayed PCT defined as parasitemia present on Day 3.
6-Miotto, 2015: Delayed PCT defined as Parasite Clearance half-life >4.5 hours (# indicates half-life <4.5 hours).

The mutations cover a 700 amino acid code region of the K-13 propeller gene, with the mutations most likely correlated with resistance appearing to be those above amino acid position number 440 [3]. The most prevalent mutation in Southeast Asia (Greater Mekong sub-region) appears to be C580Y. Other common mutations also include Y493H, R539T, and I543T [2].

These K-13 mutations have been found in both Asia and Africa. The mutations in Africa, however, have not been associated with delayed parasite clearance at this time – as the ACTs are still highly effective in Africa [3, 13, 90, 95].

Historically, mutations initiated in areas of Southeast Asia and then migrated across continents to Africa [3, 54, 55, 76]. It is not clear if artemisinin resistance will follow the same pattern migrating from Asia to Africa or if mutations or new foci are developing independently in different regions - or possibly a combination of both scenarios [77, 83]. A recent study has mapped the spread of K13 mutations across Southeast Asia and to the border of India. Though it has not been identified within India yet, this is the same historical path observed with the spread of resistance previously - and once through India on to the African continent [97].

Currently there are no validated molecular markers for artemisinin resistance and no validated test for resistance. The abundance of consistent evidence surrounding the K13 mutations however has recently lead for a call to proceed to the validation and production of a test and a harmonized approach or consortium for this effort [49, 66]. In the recent Status Report on Artemisinin Resistance, the WHO has also now added K13 surveillance as one of the potential components for ‘suspected’ artemisinin resistance; but still would require the K-13 mutation in combination with delayed parasite clearance for it to be considered as ‘confirmed’ artemisinin resistance [114].

In the absence of a validated test for resistance, clinical assessment of signs of resistance consisting of clinical observation or case reports were the initial types of evidence being reported and will continue to be monitored across these regions.

Drug resistance is often initially indicated by a delay in parasite clearance time (the time from starting treatment until no parasites can be detected); a higher treatment failure rate (anti-malarial drugs typically have only around a 5% failure rate, so an observed increase in patients failing to respond to treatment would be a cause for concern); or successful clearance followed by a return of the initial parasites (recrudescence). The latter however is more difficult to assess on a routine basis though as it may be difficult to determine if the initial parasites have returned or if a new infection has occurred without performing a more advanced molecular genotyping or PCR analysis [86].

The WHO has provided the following definition of artemisinin-resistance in their Global Report on Antimalarial Drug Efficacy and Drug Resistance: 2000-2010 shown in Table 2.2.

Table 2.2. Definition of Artemisinin Resistance

Box 6. Definition of artemisinin resistance
Only patients who meet the following criteria are classified as having an artemisinin-resistant infection: • persistence of parasites 7 days after treatment or recrudescence within 28 days after the start of treatment (artemisinin-based monotherapy), • adequate plasma concentration of dihydroartemisinin, • prolonged time to parasite clearance and • reduced in vitro susceptibility to dihydroartemisinin (Noedl, 2005). Markedly prolonged parasite clearance time (Dondorp et al., 2009). In the remainder of the document, the term 'artemisinin resistance' is a working definition used to refer to: • an increase in parasite clearance time, as evidenced by 10% of cases with parasites detectable on day 3 after treatment with an ACT (suspected resistance); or • treatment failure after treatment with an oral artemisinin-based monotherapy with adequate antimalarial blood concentration, as evidenced by the persistence of parasites for 7 days, or the presence of parasites at day 3 and recrudescence within 28/42 days (confirmed resistance).*

* This definition may be prone to confounding factors (known and unknown) such as splenectomy, haemoglobin abnormalities and reduced immunity.

Source: WHO – Global Report on Antimalarial Drug Efficacy & Drug Resistance, 2000-2010

Included in the report is the following observation that failure to clear parasites by Day 3 is likely indicative of the development of artemisinin resistance [16, 89]. The definition also allows for a determination of resistance based on 'markedly prolonged parasite clearance time' [111].

Assessing 'prolonged' or 'delayed' parasite clearance time allowed considerable variation in interpretation. Fortunately, more recently Genome-wide association studies (GWAS) used for phenotyping have been able to better define resistance from prolonged or delayed parasite clearance to a more reliable and consistent measurement of "slope half-life >5 hours" - where the slope half-life represents the time required to reduce parasites by 50% [11, 57, 65, 91, 115]. This is the current clinical definition of resistance and is the definition used for the analyses in this dissertation.

2.3. Artemisinin Resistance in Asia

High failure rates have been reported when using ACTs in areas of Asia, in particular along the Thai-Cambodian border. This is an area where drug resistant *Plasmodium falciparum* has been recorded in the past. As there have been no validated markers or tests for artemisinin resistance in the past, the confirmation of resistance was often made through clinical observation as noted above.

One of the initial studies detecting probable artemisinin resistance in the region was conducted by Noedl, et. al. in 2008 which looked at parasite clearance times along with plasma concentrations and in vivo and in vitro susceptibility to a metabolite of artemisinin (dihydroartemisinin) to assess drug level susceptibility. Patients were admitted to clinic for 28 days to ensure no new infection would compromise the assessments. Based on meeting the clinical criteria of prolonged parasite clearance time and adequate metabolite levels, they did classify 2 of 60 patients in the study as artemisinin resistant [61]. A study conducted the following year to assess the possible spread of artemisinin resistance across the region based on data of

susceptibility to dihydroartemisinin indicated the resistance appeared to be concentrated in Cambodia and had not spread across Thailand as feared. [62]

In a later study to assess if the artemisinin-resistance may be spreading, a study conducted by Phyo, et al. in 2012 also looked at the distribution of parasite clearance times over a 10-year period in patients on the western border of Thailand (Thailand-Myanmar/Burma border) to assess if there was a prolongation in clearance times with the use of artemisinin therapies over time. The study provided evidence of slowing of parasite clearance responses indicative of resistance and was consistent with that expected from a parasite population under drug selection. The genotypes were also compared between the parasites from the Cambodian region to those of the Myanmar region and were not found to be in common. However, when comparing the pair-wise allele sharing of the slow-clearing parasites vs. the fast-clearing parasites to the 'resistant' parasites in the Cambodian region, a relationship was found in the slow-clearing parasites – indicating a spreading of the resistant parasites from region to region [73].

These initial studies signalled the alarm for the possible development of artemisinin resistance. Later studies using the more advanced and reliable clinical definition of delayed parasite clearance based on slope half-life >5 hours have found consistent evidence of delayed clearance; and the supporting evidence mentioned with the recent discovery of the k-13 mutation findings have lead to the conclusion that artemisinin resistance is present in the regions of southeast Asia [2, 3, 53, 63, 92, 96].

Artemisinin resistance defined as delayed parasite clearance time following an ACT does not however necessarily lead to overall treatment failure or recrudescence. The partner drug may still confer benefit and allow successful outcome [114].

Ittarat, et al. conducted one of the first studies to investigate risk factors for treatment failure or recrudescence with artemisinin therapy. The study was conducted in Thailand and

studied artemisinin monotherapy. ACT/combination therapy was not included. The study concluded the recrudescence or treatment failure in this setting was not due to parasite resistance and was dependent on parasite burden at baseline [38]. This was consistent with an earlier finding by Price et al. showing parasitemia at baseline as a significant risk factor for recrudescence/treatment failure [72].

Stepniewska, et. al. investigated in vivo parasitological measures of artemisinin susceptibility. Using pooled data from ACT containing anti-malarial drug studies in uncomplicated falciparum malaria, the objective was to determine the effects of various covariates on therapeutic response. The covariates included: transmission intensity, age, and parasite density. The study found that assessing parasitemia at 72 hours (day 3) was a good predictor of whether or not a patient would have recrudescence. In addition, this research appeared to support the earlier conclusions from Ittarat that the main factor affecting initial parasite clearance time in this data was baseline parasite burden [89].

Vijaykadga et al. investigated the use of artesunate-mefloquine therapy in the treatment of uncomplicated *Plasmodium falciparum* in Thailand from 1997-2007. A retrospective study was conducted to compare parasite clearance times over time to assess prevalence of parasitemia following treatment and to investigate if delays in parasite clearance may indicate artesunate resistance. The study did find delayed parasite clearance times over this period indicating early artesunate susceptibility. The study also found the inability to clear parasites by day 2 or day 3 was not a good indicator of overall treatment failure or recrudescence (e.g. at Day 28) [99].

2.4. Artemisinin Resistance in Sub-Saharan Africa

ACT resistance in SSA has not been documented yet. However, there have been cases or pockets where slowed clearance times have caused concern [3]. While emergence of resistance in

other parts of the world may be tolerable, if ACT resistant malaria reaches the African continent it could be a dire situation [67].

Even though slowed parasite clearance time can be an indicator of resistance, it is not the only cause. It could be due to inadequate drug levels or exposure – which may be due to inadequate dosing or even the possibility of non-potent or fake/fraudulent medication [3, 6, 7, 17]. It is also possible that slowed clearance may appear not due to resistance, but due to natural immunity that may have decreased in a region due to effective malaria control in the past [9, 31]. Other possible determinants of parasite clearance time in this region include age, previous exposure/episodes of malaria or transmission as surrogates for the development of immunity [19, 29, 33, 60].

Some studies have been conducted to investigate whether artemisinin resistance appears to be spreading to SSA. These studies are often limited to a small number of patients or data for a single country or center for investigation. Also, they often have not used standard methods to assess PCT. Data from combined studies have also been presented, but they often include studies with differing designs, collection methods and timepoints which hinder the assessment of PCT.

To date the study research has not indicated the spread of resistance in this region. However, clinical reports of treatment failure continue to arise. Also, with the history of the spread of resistance from Asia to SSA for previous therapies, this is still a concern of utmost importance to keep in the forefront [67].

Sowunmi et al. investigated the factors contributing to the delay in parasite clearance by combining data from Randomized Controlled Trials (RCT) studies in Nigeria from 1996 to 2008 to investigate any changes in parasite clearance over time. ACT and non-ACT regimens were examined. Though changes in PCT were obvious over time in the non-ACT therapies, the performance of the ACT therapies did not appear to indicate current resistance. However, delays

in PCT were associated with increased gametocyte carriage and the possibility of transmission of drug resistant phenotypes, and this finding was applicable to both the non-ACT and the ACT therapies [88].

Maiga et al. investigated the use of artesunate monotherapy in the treatment of uncomplicated *Plasmodium falciparum* in Mali. A prospective study was conducted in 100 children to assess PCT and treatment failure in 2010-2011 with results compared to a similar population at the same clinic in 2004. Though there were limitations to the study, there was no indication of decreased PCT or the development of artemisinin resistance in this population had developed over time [46].

Gbotosho et al. combined data from 6 randomized controlled trials and one open-label trial in Nigeria to assess changes in efficacy and parasite clearance times over a 5 year period for patients treated with two ACTs (AA=amodiaquine artesunate and AL=artemethur lumefantrine). Data for 811 subjects were assessed for clinical measures (n=210, 2005; n=206 in 2008; n=285 in 2009; n=110 in 2010). However, serial measures to assess parasite clearance time half-lives were only available for less than half the subjects. Parasitological efficacy remained unchanged over the 5 year period and no concern for drug resistance was noted. As noted with other studies, parasite clearance was associated with baseline parasitemia. The half-life was 1.09 hours with no difference based on age or treatment. Recrudescence was rare (n=18, 2.2%) and was not associated with treatment or age [30].

Borrmann et al. compared PCT data for 474 children in an out-patient RCT of two ACTs (DHA-PPQ - dihydroartemisinin-piperaquine and AM-LM – artemether-lumefantrine) conducted in 2005-2008 in Kenya to assess if subjects enrolled in the later two years of the study had delayed PCT compared to those enrolled in the first two years. A small but significant decrease in clearance time was detected in the study. This was attributed to a reduction in population-level

immunity due to decreased transmission levels in the study area, and not likely due to resistance [9]. Similar findings were reported by Greenhouse et al. from an earlier study of n=129 subjects in Kenya [31].

Dorsey et al. investigated age, temperature, and parasite density on treatment outcomes in anti-malarial studies in Uganda (prior to the introduction of ACTs), by combining data from 6 previous trials of 7 standard therapies. They found patterns of decreasing age, increasing temperature and parasite density predicted decreasing treatment response suggesting the failures can be explained by factors other than resistance [19]. Similar findings for age, temperature, and parasite density were noted by Hamer et al. in a multi-center study of Chloroquine treated subjects in countries in central Africa and Ecuador [33].

Francis et al. investigated whether differences in treatment response and treatment failures in Uganda were due to anti-malarial drug resistance. Molecular markers of drug resistance were available for the therapies under study (non-ACT therapies). This was conducted prior to the use of ACTs and with reliable molecular markers of resistance for the current therapies available. Their findings suggested geographical differences (transmission intensity / endemicity) not parasite factors explained the differences in response [29].

Finally, as noted earlier, the recent findings with the association of the K13 (kelch) mutations have also been explored for data in sites in Africa (Kenya, Nigeria, and Democratic Republic of Congo). Parasite clearance half-lives >5 hours were identified in some patients, however, they were not associated with K13 mutations. Also, there were three separate patients identified with K13 mutations, however, these patients had rapid parasite clearance so these mutations were not associated with delayed parasite clearance [3]. Similar findings were noted with other recent studies in SSA [13, 95].

It is not clear if artemisinin resistance is developing in SSA and if so if it will follow the patterns observed in Asia or if it will emerge as something slightly or entirely different.

2.5. Assessment of Parasite Clearance Time

Parasite clearance time (defined as the time from dosing to the clearance of all asexual parasites from the blood) was often used to assess the effectiveness of anti-malarial drugs; and a slowing of the time to clearance used as a signal to confer resistance. It is a useful measure however, an understanding of the issues inherent in the measure and the implications is important. For example, it can be an imprecise measure due to random and systematic errors in measurement [105]. The time to clearance is also often directly impacted by the initial parasitemia which may have more or less of an impact depending on the stage of disease – e.g. uncomplicated *P.falciparum* malaria is more stable, however, severe malaria where parasitemia is at very high levels would likely be more impacted by the baseline or starting parasitemia. The frequency of assessments or sample collection (multiple times per day vs. daily) may also impact the precision of the measurement [28, 39]. In regions where clearance is typically fast, an initial delay between 24-48 hours may be an indication of reduced susceptibility or resistance; however only assessing whether full clearance has occurred at 72 hours (3 days) may not detect this important delay or change [25].

A preferred measure of anti-malarial effect as noted by Flegg, et. al. in the discussion for standardizing the measure of parasite clearance is the slope of the log-linear portion of the parasitemia curve versus time. The measurement of parasite clearance time can be complicated by a potential for a variable lag in the initiation of the curve which can introduce inaccuracies in assessing the time from initial parasitemia. There is also the potential for tail measurements which may contain variability when estimating the actual clearance time. Using the slope could offer a more consistent and robust estimate of the treatment effect [17, 27].

Nick White in his paper 'The parasite clearance curve', describes the traditional parasite clearance time measurement as a useful measure of anti-malarial drug-effect, however, with limitations due to the issues with imprecision and the dependency on the baseline parasite level. A visualization of the dependency on the baseline parasite level is shown in Figure 2.1 from White, 2011 – where two patients with different baseline parasite counts (shown on the y-axis), experiencing the same therapeutic benefit from an anti-malarial treatment could have very different parasite clearance times (shown on the x-axis) based on the traditional calculation of time from dosing to clearance of all parasites (below the limit of detection) [105].

Figure 2.1. Parasite Clearance Curve

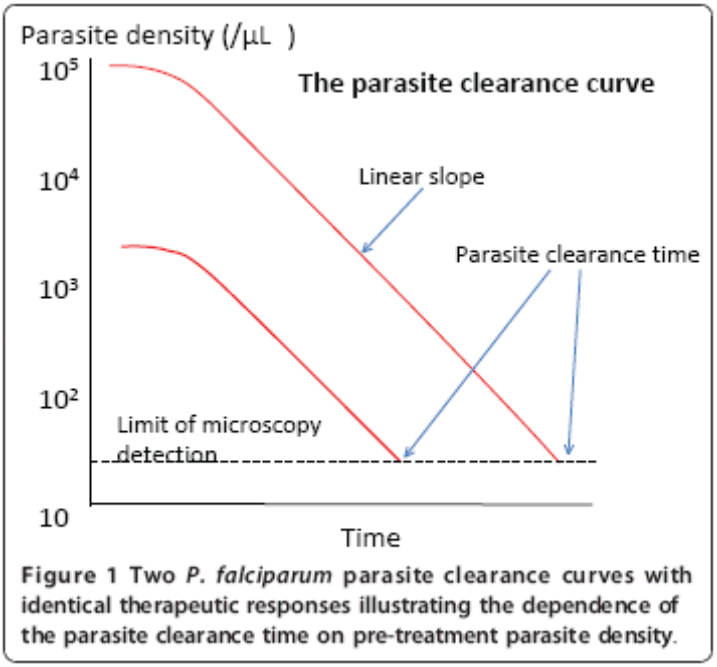


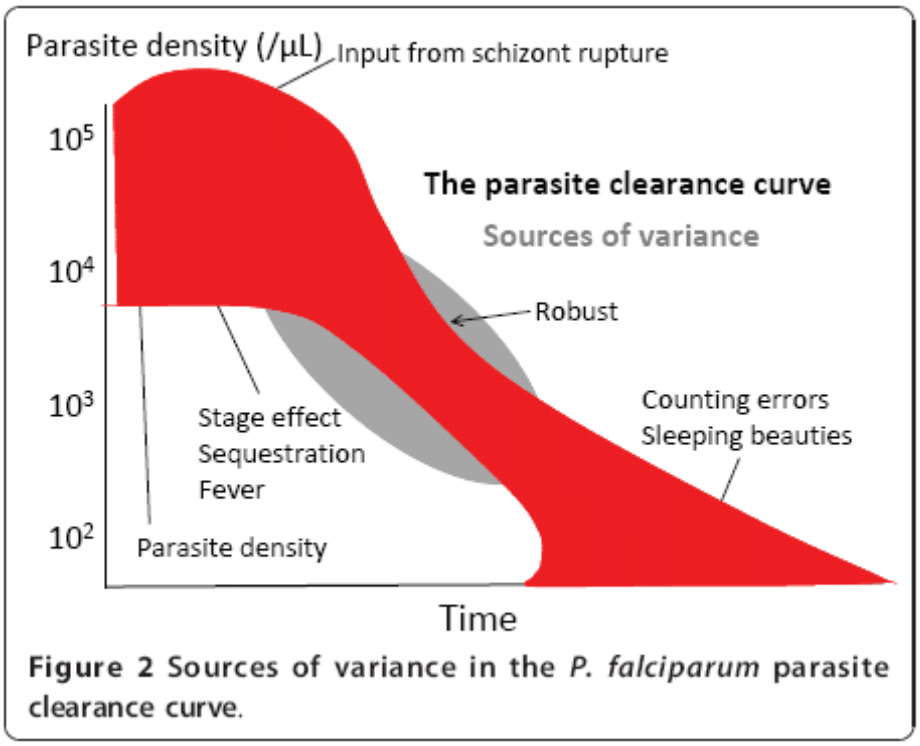
Figure 1 Two *P. falciparum* parasite clearance curves with identical therapeutic responses illustrating the dependence of the parasite clearance time on pre-treatment parasite density.

Source: The Parasite Clearance Curve – N. White, 2011

A more robust estimator of therapeutic effect (or "lack of effect" or potential for resistance) would be derived from the middle portion of the parasite clearance curve (e.g. the linear slope). This is shown in the Figure 2.2 below from White, 2011 – where the sources of variation which impact the precision or ability to assess the therapeutic effect are removed from the measure. Examples of this variability include the initial increase in parasitemia that may occur when the

initial fever outbreak occurs and a patient seeks treatment, e.g. the disease stage effect seen early on (the early part of the curve); and the counting errors which may occur at the later stage of assessment when parasitemia is very low and there is a need to move from thin to thick blood slides to obtain a more accurate assessment of clearance or values going below a limit of detection (the later part of the curve) [105].

Figure 2.2. Sources of Variance in the Parasite Clearance Curve



Source: The Parasite Clearance Curve – N. White, 2011

In order to address differences in measures and to standardize and obtain a consistent approach to identifying potential resistance, a parasite clearance estimator (PCE) tool was developed by the Worldwide Antimalarial Resistance Network (WWARN) [119]. It provides a consistent algorithm for removing the lag portions and tail portions of the curve and calculations for the slope. This would also allow a consistent approach for all malaria studies to use and enhance comparability across studies and the ability to more accurately detect changes in clearance times. The Parasite Clearance Estimator as described by WWARN "provides a consistent,

reliable, and accurate method to estimate malaria parasite clearance based on the linear portion of the slope of the log-parasitemia versus time relationship. This standardized approach will facilitate routine monitoring of ACT efficacy and allow comparisons over time and space" [119].

The discussion in the literature to move to this new standard measure of slope half-life to assess parasite clearance and the new PCE tool have only recently been made available since 2011, so studies conducted previously would typically not have had access to this measure for reporting. However, the standardized approach will offer an advantage for comparability of future studies and a more precise way to assess changes in PCT which may be indicative of resistance going forward [82, 118].

In addition to providing measurements of the Parasite Clearance Time and the slope half-life, the parasite clearance estimator tool also provides traditional measures of PC50, PC95, PC99 (time when 50%, 90%, 95%, 99% of the parasites have cleared and estimates of the lag time [119].

All of these measurements together are useful to build a profile to assess parasite clearance and look for changes or prolongation of clearance time which is a critical way to assess artemisinin-resistance in the current environment.

2.6. Assessment of Recrudescence

Recrudescence is an indication of treatment failure and possibly resistance. This arises when a return of the original parasite infection occurs after treatment with an anti-malarial therapy. One of the difficulties in assessing recrudescence is determining if a recurrence or return of a positive parasite count after treatment is due to a return of the original parasites (recrudescence) or a new infection (re-infection due to a new mosquito bite) or possibly a combination of both (mixed infection) [86].

In order to assess if recrudescence has occurred, molecular genotyping is undertaken to distinguish recrudescences from new infections. Polymerase chain reaction (PCR) amplification is

used to assess if the genes from the parasites in the original infection at baseline are also observed when the parasite count becomes detectable again after treatment. In the past, the number of genes assessed and methods varied leading to inconsistencies in reporting across studies. Now standardized approaches based on WHO protocol are used across anti-malarial studies which include the testing for polymorphisms in three particular genes: merozoite surface protein 1 (msp-1), merozoite surface protein 2 (msp-2), and glutamate-rich protein (glurp) [12, 109]. If a subsequent sample contains identical alleles or a subset of alleles present in the baseline sample the infection is considered to be a recrudescence. If the sample contains only new alleles it is considered to be a new infection (re-infection). If a sample contains both it is considered to be both a recrudescence and a new infection (mixed infection). Results which contain both (mixed infection) are typically classified as treatment failures since any recrudescence would indicate all of the original parasites were not eliminated; however, differing summary or analysis methods may be have been used [10, 111].

In addition to the molecular genotyping, the timing of assessments has also now been standardized for studies conducted with the WHO protocol [107]. In the past, testing typically started on Day 7 and did not go beyond Day 14 – so any recurrence beyond this point was assumed to be a new infection and not indicative of a failure of the previous treatment. With the use of ACT therapy, a longer follow-up period was needed to assess the possibility of recrudescence occurring. The length of testing has been standardized so testing typically starts at Day 7 and now continues beyond day 14 to day 28 or day 42 depending on the half-life of the drugs to ensure an adequate follow-up period has been assessed. [110]

Even with the standardized protocols for testing for recrudescence, there still are inconsistencies and issues which may impact the accuracy of these assessments. For example, protocols may use the same 3 genes for testing but allow for a different range of base pairs when

assessing for identical alleles or subsets of alleles. This variability could cause differences in the classification or misclassification due to false positive or false negative detection of recrudescence. It is possible that a new infection may have occurred but with a parasite possessing similar genetic makeup to the baseline parasite. In this case it may have been erroneously misclassified as a recrudescence (false positive). Similarly, it is possible a genotype may have been present in the baseline sample, but at an undetectable level. When comparing the post-treatment sample it may appear that a new infection has occurred, when it actually is a recrudescence of a previously low-level or infrequent variant (false negative) [14, 69].

In the analysis stage, different approaches may also be used which could cause the failure rate (recrudescence rate) to appear to vary as well. For example, cases with recrudescence plus new infection (mixed cases), could be classified as indeterminate and excluded from the analysis rather than being considered as treatment failures or could even be classified a non-recrudescence (technically including them as a new infection) [86]. The analysis method could have a dramatic impact on the reported recrudescence rate and it is important to look closely at the method used for summarizing the mixed cases when comparing results in the literature.

Though these issues do exist, the movement toward standard protocols based on WHO guidelines is improving and the assessment of recrudescence is more consistent in studies incorporating the genotyping for recrudescence. The use of genotyping is now routinely included in clinical trials studying the therapeutic effect of ACTs. However, the assessment of treatment failure due to recrudescence outside of a clinical trial setting, e.g. for individual cases or in a clinical practice setting, is not typically available and may impact surveillance efforts [94].

2.7. Influence of Host Factors vs. Parasite Factors

A delay in parasite clearance time (slope half-life >5 hours) is an indicator of resistance. In the absence of an assessment of validated genetic markers to confirm parasite factors, delayed clearance times are attributed to resistance and are used to define resistance.

For uncomplicated *Plasmodium falciparum*, parasite clearance can typically be influenced by baseline parasitemia, natural and/or acquired immunity, frequency of measurements, drug exposure, and resistance [1, 25, 82]. Erythrocytic polymorphisms have also recently been found to influence parasite clearance [1]. It is important to also understand factors that influence parasite clearance may not be the same in all regions. For example the influence of age on immunity and clearance occurs in SSA where acquired immunity is common, but age is not a significant factor in southeast Asia where malaria is less frequent [1, 25].

Host (human) factors, immunity in particular, may contribute and influence the ability to clear parasites [1, 15, 22, 25, 41, 45, 82]. Humans with a first exposure to malaria will most likely become ill. Older children and adults in endemic areas develop protection from illness and death [26, 103]. Immunity does wane if leaving and then returning to an endemic area. However, the immunity does appear to quickly return [43]. This acquired immunity from multiple malaria exposures enables individuals to clear infection without drug treatment. This acquired immunity also adds a complexity when attempting to assess drug resistance in these cases - since these individuals when treated with anti-malarial therapy may clear the parasites including the drug-resistant parasites. In these cases the ability to clear the parasite would likely be attributed to the drug therapy.

Protective immunity as seen with age, shows similar results where increasing age encourages increasing protective immunity and decreasing treatment failure [8, 45]. Similarly, older children and adults receiving anti-malarial therapy may still effectively clear even the

resistant parasites [41]. Of note, very young infants also appear to have initial protective immunity conveyed from their mothers, but this appears to last less than 6 months. [24, 74]

In addition to acquired immunity and age-related immunity, transmission intensity (or endemicity level) also enhances immunity – with higher transmission intensity hastening the development of immunity, due to more frequent exposure to malaria parasites. Treatment failure is also less common in high transmission areas [5, 24, 29, 74, 93].

Immune responses from malnutrition, co-infection with HIV, and pregnancy all pose similar relationships with the susceptibility to malaria increased – although patterns are not as conclusive for these cases [58, 74]. A higher temperature at presentation may also be a surrogate for a less effective host immune system response to infection [33]. However, this has been difficult to study due to limited data and limited range of temperature data at presentation [19].

2.8. Summary

The efficacy and safety of malaria treatments have been studied in depth, with thousands of patient exposures reviewed for regulatory applications and research. Most therapies have very high efficacy rates (>95%) and safety profiles that are acceptable in the benefit:risk setting for malaria.

Information on patients with delayed or slow parasite clearance or patients who later develop recrudescence however is less well known. Studies are not routinely conducted in-clinic or with frequent assessment times to determine accurate assessments of parasite clearance. In addition, clinics may monitor patients for recurrence of an infection, but they typically do not assess whether the recurrence is due to a recrudescence (return of the original parasites) or a new infection.

The possibility of resistance remains a concern; and control will be difficult without knowing which areas may have failures due to changes in immunity and which areas may have true artemisinin resistance [4, 52].

With very few treatment options on the horizon, any spread of artemisinin resistance to SSA would be a devastating situation. Now is the time for diligence in monitoring for any potential spread of artemisinin-resistant strains to SSA.

3. Limitations of the current study data

As shown above, data on the exploration of artemisinin resistance in SSA is very limited. Resistance in Asia was only recently confirmed [61, 73] and until very recently there were no biomarkers or laboratory tests to confirm artemisinin resistance. Investigation until just recently (2013/2014), has often been limited to the guidance as noted in the WHO documentation to assess the potential for artemisinin resistance based on prolonged parasite clearance times and increased treatment failure rates and recrudescence [106, 108, 110, 112, 114].

Though many studies have been conducted in Asia and a clearer understanding of the profile of resistance in this region is available, similar research in SSA is very limited. Where studies have been conducted in SSA, they are often small studies or are limited to a single country or center.

Meta-analyses and investigations conducted comparing parasite clearance times across studies conducted at different times are notably subject to differences in data collection methods, differences in sample collection times, and differences in analytical approach which may lessen the strength or interpretation of the comparison.

Until recently, the clinical definition of resistance was delayed parasite clearance time, without any indication of a threshold for assessment. The GWAS studies have provided support

for a more formal clinical definition of slope half-life > 5 hours. Since this was only made available in 2014, however, the previous studies would not have used this more robust definition.

Very few studies have also used the Parasite Clearance Estimator tool available from WWARN, as this was only recently made available for widespread use. It will be interesting to see how the implementation of the standards for parasite clearance estimation will impact the assessment of changes in PCT and comparability across studies in the future.

Several limitations have been noted regarding the previous study data available for SSA. The current analyses in this dissertation offered a unique opportunity to better understand parasite clearance data and recrudescence data for ACTs in SSA by utilizing data previously collected from a large multi-center and multi-national study in SSA and to explore determinants of PCT and recrudescence while applying current definitions and tools which may be helpful to better assess the determinants of PCT and recrudescence.

4. Parent Study Data

One of the established gold-standard ACTs for treating malaria in SSA is Coartem (artemether-lumefantrine). As new therapies are proposed they are compared to this gold-standard therapy to assess efficacy and safety. New ACTs are however few and far between. In order to be a candidate, the original therapy must have a good efficacy and safety profile; as well as a good resistance profile and would be complemented by the addition of an artemisinin. One such candidate was CDA (chlorproguanil-dapsone-artesunate). A full-scale registration program was planned to study this combination. A large phase III study was conducted in SSA. The study included a head-to-head comparison of artemether-lumefantrine with chlorproguanil-dapsone-artesunate (n=1372). The study was conducted in children (1-14 years) with an in-clinic setting for 3 days including extensive parasite monitoring; then follow-up for 42 days to assess recurrence.

The study was a multi-center and multi-national randomized controlled trial (RCT) conducted in SSA and following Good Clinical Practice (GCP) and Good Laboratory Practice (GLP) standards. Patients and/or guardians were consented prior to the study and Disease History and Baseline Results were documented. The study collected parasite count data for 3 days, with measurements every 8 hours. The study also included a follow-up period with genotyping of any recurrences using the more comprehensive genotyping of MSP1, MSP2 and GLURP to assess recrudescence vs. new infections. The study was also setup and followed the WHO Protocol and utilized the WHO definitions for assessing response outcomes [107].

The study was conducted with full enrolment; ran to completion; and was reported. The study met the efficacy and safety objectives. However, due to a safety concern in a subset of patients with G6PD-deficiency (glucose-6-phosphate dehydrogenase) which exposes these patients to a greater risk of having a hematologic event, the registration of this product did not go forward.

The study collected extensive treatment data on ACTs in a large multi-center, multi-national setting in SSA. It provides a unique opportunity for additional data exploration which may offer useful insight into possible determinants of parasite clearance time and recrudescence or treatment failure with ACTs in regions in SSA.

Design of the Parent Study

The parent study is a double-blind, multi-center RCT designed to assess efficacy in patients treated with chlorproguanil-dapsone-artesunate (CDA) vs. the artemether-lumefantrine (AL). 1372 patients, age 1 to 14 years, were randomized in a 2:1 ratio (914 CDA : 458 AL), to allow additional safety data to be collected on the new treatment. The study was conducted from June 2006 to August 2007 in the following regions in SSA: Burkina Faso, Ghana, Kenya, Nigeria, and Tanzania.

Patients eligible for the trial had: uncomplicated *P.falciparum* malaria infection of 2,000 – 200,000 parasites / uL and were aged 12 months to 14 years old. Patients were not eligible if they had severe/complicated malaria; or had a known history of G6PD deficiency. The specific study inclusion/exclusion criteria are shown below:

Inclusion Criteria:

- Acute, uncomplicated *P. falciparum* malaria, microscopically confirmed
- Temperature at screening 37.5^{o}C or more or confirmed history of fever within the previous 24 hours
- Weigh 7.5kg or over
- Screening hemoglobin (Hb) of 7g/dl or over, or hematocrit of 25% or more (if Hb not available at screening)
- Willingness to comply with the study visits and procedures, as outlined in the informed consent form
- Written or oral witnessed consent has been obtained from parent or guardian
- Assent is given by a child aged 12 years or over, in addition to the consent of their parent or guardian

Exclusion Criteria:

- Features of severe/complicated malaria
- Hypersensitivity to active substances (chlorproguanil, dapsone, artesunate, artemether lumefantrine)
- Known allergy to biguanides, sulphones, sulphonomides, artemesinin derived products or aminoalcohol drugs
- Known history of G6PD deficiency
- Infants with a history of hyperbilirubinemai during the neonatal period

- Use of concomitant medications that may induce hemolysis or hemolytic anemia from the WHO (World Health Organization) list of essential drugs
- Evidence of any concomitant infection at the time of presentation (including P. vivax, P. ovale, and P. malariae)
- Any other underlying disease that may compromise the diagnosis and the evaluation of the response to the study medication (including clinical symptoms of immunosuppression, tuberculosis, bacterial infection; cardiac or pulmonary disease)
- Malnutrition, defined as a child whose weight-for-height is below -3 standard deviations or less than 70% of the median of the NCHS/WHO normalized reference values
- Treatment within the past three months with mefloquine or mefloquine-sulfadoxine-pyrimethamine; twenty-eight days with sulphadoxine/pyrimethamine; sulfalene/pyrimethamine, lumefantrine or artemether/lumefantrine, amodiaquine, atovaquone or atovoquone/proguanil, halofantrine; 14-days with chorproguanil-dapsone, or 7-days with quinine (full course), proguanil, artemesinins, tetracycline doxycyline or clindaycin
- Positive sulphadoxine/pyrimethamine urine screen for ‘unknown’ antimalarial drug use in prior 28 days
- Use of an investigational drug within 30 days or 5 half-lives whichever is the longer
- Previous participation in this study
- Female subjects of child-bearing age, who have had a positive pregnancy test at screening, or do not give their consent to take a pregnancy test
- Female subjects who will be breast-feeding an infant for the duration of the study

Baseline assessments included: temperature, age, height (or length); prior and current medical history; current medications; history of previous malaria episodes in children <= 2 years; and a baseline sample for assessment of G6PD status. Hematologic testing included hemoglobin, hematocrit, methemoglobin (when available), and counts of red blood cells (RBCs), reticulocytes (expressed as proportion of total RBCs), white blood cells (WBCs), and platelets. Clinical chemistry parameters included serum creatinine, total and indirect bilirubin, aspartate aminotransferase (AST), and alanine aminotransferase (ALT).

Patients were treated in-clinic for 3 days and then followed-up at visits on day 7, 14, 28 and 42. At each visit, samples were taken to assess parasite type and counts and to perform laboratory evaluations for clinical chemistry and hematology. Assessments for adverse events occurred at each visit and also were updated if reports were made between visits. Further details of the study are available in the US-NIH Clinical Trials Registry [98].

Parasite Clearance Time

Asexual parasite and gametocyte counts were performed at screening, at pre-dose, every 8 hours during the in-patient stay until discharge on Day 3 and at follow-up visits on Days 7, 14, 28 and 42. At each time point, two thick and one thin film were prepared and parasite densities determined by examination of a thick blood slide (10 ML thumb prick), according to WHO methods [107].

Parasite clearance time was assessed as the time needed to clear asexual parasite forms from the blood; parasite numbers fall below the limit of detection in a thick blood smear and remain undetectable for at least 48 hours.

In this study, asexual parasite counts decreased rapidly in both treatment groups. By 16 hours post first treatment dose parasite counts had declined by > 99% in both groups. Mean (SD) parasite clearance time was 23.5 (11.0) hours for CDA and 26.2 (11.5) hours for AL [70].

Recrudescence

For the Parasite DNA analysis - two drops of peripheral blood were collected onto pre-printed filter papers, for subsequent DNA extraction and PCR analysis of *P.falciparum* DNA, on all subjects at screening and any day on or after day 7 when a blood slide is prepared. PCR of the *P.falciparum* genes MSP-1, MSP-2 and GLURP were used to distinguish between the initial infection reappearing (recrudescence) and a new infection occurring on any day on or after day 7 in the 42-day follow-up period.

For the reported analysis of adequate clinical and parasitological response at 28 days 93% and 94% of patients responded and at 42 days 90% and 93% responded with CDA and AL. Patients with new infections and no sign of recrudescence were not considered to be failures. Patients with recrudescence or recrudescence plus new-infection were considered failures [70].

CHAPTER 3: RESEARCH DESIGN AND METHODS

The current research proposal is an ancillary analysis of the parent Randomized Controlled Trial (RCT) described above. The parent RCT was designed to assess efficacy and safety of a new ACT. The ancillary analyses described in this dissertation were conducted to investigate the potential determinants of parasite clearance time [Study Aim 1] and determinants of recrudescence [Study Aim 2] that may be useful in addressing if treatment failure is due to host factors such as immunity; or parasite factors which might convey signs of the development of artemisinin resistance in SSA.

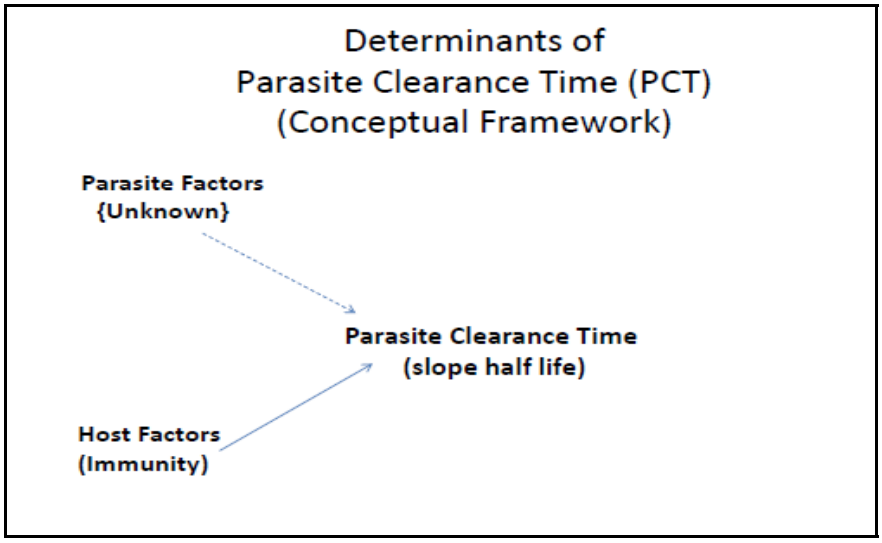
1. Study Aim 1 - Determinants of Parasite Clearance Time

A slowing of parasite clearance time is potentially an indicator of resistance, but could also be a sign of individual or population changes due to natural or acquired immunity or some other explanation.

An exploratory analysis of determinants of parasite clearance time was planned to assess if there are other factors which might predict a slow or elongated parasite clearance time.

The following Conceptual Framework shown in Figure 3.1 describes the relationship being explored.

Figure 3.1. Determinants of Parasite Clearance Time



1.1. Outcome of interest

The primary outcome for this analysis is parasite clearance time assessed by the slope half life of the parasite clearance curve. The slope half life represents the estimated time in hours for parasitemia to decrease by half. The calculation utilized the Parasite Clearance Estimator (PCE) software available from the WWARN website to allow consistency in reporting and assessing potential malaria resistance [119].

1.2. Covariates of interest

The following covariates were considered for evaluation as surrogates for host immunity:

Age – based on natural immunity patterns over age range

- <5 years
- >=5 years

Presence of Previous Malaria Episode and Number of previous malaria episodes

Seasonality

- Rainy
- Dry

Estimated Endemicity

- <60%
- >=60%

Geographic Location (Study Center or Region to assess transmission intensity)

- Burkina Faso
- Ghana
- Kenya
- Nigeria
- Tanzania

The selection of covariates is based on factors associated with host immunity in the literature. Other covariates included baseline parasitemia, sex, and treatment group.

1.3. Data collection methods

Data collection and validation for the parent study was conducted under SOPs for Good Clinical Practice (GCP) and to Good Laboratory Practice (GLP) standards. Data were collected on a Case Report Form (CRF) that was pre-tested for readability and accuracy in capturing planned data. Data from the CRF were then entered into the sponsor's database where additional validation and consistency checks were run. Missing data points were queried as well as any results that were inconsistent across the CRF. Consistency checks included both a manual clinical review of the CRF information as well as programmed validation checks.

Once the data quality assessments were complete the study database was authorized for analysis.

For the current ancillary study, the database is already locked and no further changes can be made. Additional investigation for outliers as well as assessing the impact of missing data however would be explored. Sensitivity analyses were undertaken using different methods of imputation if missing data was problematic. Missing data from the primary study was not anticipated to be a concern for this study as it was conducted in-clinic or on a controlled out-patient setting so follow-up was monitored throughout. The primary analysis included only available data for the assessments.

1.4. Data analysis methods

The statistical models used to estimate the parasite clearance measures and lag phase duration were fitted using the Parasite Clearance Estimator (PCE) developed by the World Wide Antimalarial Resistance Network (WWARN) [119].

The analysis for Parasite Clearance Time with outcome assessed as the slope of the parasite clearance estimator is calculated as:

- *Slope half life = $T_{½} = \log_e 2 / K = 0.692/K$,*

  where K is the clearance rate constant.

- *Clearance rate constant is calculated from the subset of data*

  after the tail and lag phase are removed,

  and utilizes the formula: $\log_e(P_t)=\log_e(P_0) – Kt$

Logistic regression was used to assess the outcome, parasite clearance time assessed by the slope half life (<= 5 hours vs. > 5 hours) and the covariates: baseline parasitemia, sex, treatment and the surrogates for immunity (age, seasonality, estimated endemicity, number of previous malaria episodes, and geographic region).

Univariate analyses were conducted to assess the association between parasite clearance time and each of the covariates individually. A multivariate logistic regression analysis was also conducted which included the full set of candidate variables with a stepwise approach using a generous selection criterion of 0.20 for entry and 0.25 to retain the variable in the model.

Sensitivity analyses were also undertaken to explore the missing data for the slope half-life assessment from the Parasite Clearance Estimator (PCE).

2. Study Aim 2 - Determinants of Recrudescence

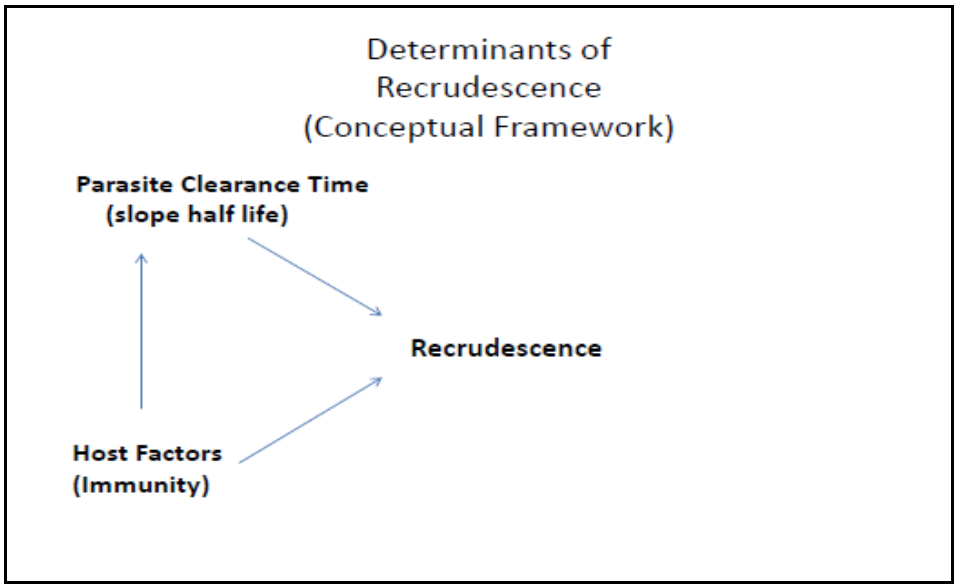
ACT therapy in SSA typically provides a high response rate >= 95%. However, treatment failure does occur and an increase in this failure rate (due to recrudescence or return of the original

parasites after initial parasite clearance) could be a concern that artemisinin-resistance may be spreading to SSA.

The aim of this analysis was to explore the determinants of recrudescence and better understand if they may be related to host factors (such as changes in immunity) or parasite factors (which could indicate resistance).

The following Conceptual Framework shown in Figure 3.2 describes the relationship being explored.

Figure 3.2. Determinants of Recrudescence



2.1. Outcome of interest

The primary outcome for this analysis was recrudescence defined as a return of initial parasites either alone or in combination with a new infection. This was assessed for each patient as a binary outcome 1=recrudescent or 0= non-recrudescent. The determination of recrudescence status was based on the PCR data reported in the parent study which assessed MSP-1, MSP-2, and GLURP to identify parasites from the initial infection vs. parasites not identified previously and therefore indicative of a new infection.

Analyses of any recurrence of parasitemia (recrudescence only, recrudescence plus new infection, and new infection only) and the time to recurrence were also explored.

2.2. Covariates of interest

The following covariates were considered for evaluation as surrogates for host immunity:

Age – based on natural immunity patterns over age range

- <5 years
- >=5 years

Presence of Previous Malaria Episode and Number of previous malaria episodes

Seasonality

- Rainy
- Dry

Estimated Endemicity

- <60%
- >=60%

Geographic Location (Study Center or Region to assess transmission intensity)

- Burkina Faso
- Ghana
- Kenya
- Nigeria
- Tanzania

The selection of covariates is based on factors associated with host immunity in the literature. Other covariates included baseline parasitemia, sex, and treatment group. In order to explore if there was an association between parasite clearance time and recrudescence, slope half life was also included in the analyses.

2.3. Data collection methods

Data collection and validation for the parent study were previously described in Section 1.3.

2.4. Data analysis methods

Logistic regression was used to assess the outcome (recrudescent vs. non-recrudescent) with Parasite Clearance Time (Slope half-life), baseline parasitemia, sex, treatment, and the covariates which are surrogates for immunity (age, seasonality, estimated endemicity, number of previous malaria episodes, and geographic region).

Univariate analyses were conducted to assess the association between recrudescence and each of the covariates individually. A multivariate logistic regression analysis was also conducted which included the full set of candidate variables with a stepwise approach using a generous selection criterion of 0.20 for entry and 0.25 to retain the variable in the model.

In addition, analyses to describe the occurrence and timing of any recurrence (recrudescence only; recrudescence plus new infection; and new infection only) were summarized.

3. Sample Size considerations

Since the study is based on ancillary analyses of an RCT, the proposed analyses are limited by the sample size of the parent study (n=1372).

All statistical analyses for this dissertation are performed post-hoc. No formal sample size calculations were considered for the analyses. The sample size was planned based on the original clinical trial objectives and reported previously [70].

An example of the associated power shown in Table 3.1 was prepared using PASS (version 12) software for a logistic regression analysis assuming a sample size of n=1372 with varying scenarios for the Odds Ratio and the percentage with response to explore the power associated with different scenarios [36].

Table 3.1. Sample size and Power

N	%N (X=1)	P0	P1	Odds Ratio	Alpha	Power
1372	2	0.05	0.1	2.11	0.05	0.28
1372	5	0.05	0.1	2.11	0.05	0.46
1372	10	0.05	0.1	2.11	0.05	0.64
1372	20	0.05	0.1	2.11	0.05	0.83
1372	40	0.05	0.1	2.11	0.05	0.93
1372	2	0.05	0.15	3.53	0.05	0.59
1372	5	0.05	0.15	3.53	0.05	0.84
1372	10	0.05	0.15	3.53	0.05	0.97
1372	20	0.05	0.15	3.53	0.05	>0.99
1372	40	0.05	0.15	3.53	0.05	>0.99
1372	2	0.05	0.20	4.75	0.05	0.80
1372	5	0.05	0.20	4.75	0.05	0.97
1372	10	0.05	0.20	4.75	0.05	>0.99
1372	20	0.05	0.20	4.75	0.05	>0.99
1372	40	0.05	0.20	4.75	0.05	>0.99

This example (for data row 7) shows a logistic regression with a binary response variable (Y) on a binary independent variable (X) with a sample size of 1372 observations (of which 95% are in the group X=0 and 5% are in the group X=1) achieves 84% power at a significance level of 0.05 to detect a change in Prob(Y=1) from the value of 0.05 to 0.15 corresponding to an odds ratio of 3.53.

CHAPTER 4: RISK FACTORS FOR DELAYED PARASITE CLEARANCE TIME

1. Introduction

The World Health Organization estimates there are 198 million malaria cases annually, causing over half a million deaths and 453,000 deaths in children under 5 years in 2013, with 90% of all malaria deaths occurring in Africa [113].

P. falciparum malaria is a common cause of mortality in young children throughout sub-Saharan Africa (SSA) and leads to significant morbidity across all age groups. However, many of the previous first line monotherapy drugs for treating uncomplicated malaria are compromised by parasite resistance. In 2005, the World Health Organization (WHO) advocated artemisinin combination therapies (ACT) as first-line therapy to forestall resistance. Any sign of resistance to artemisinin in SSA would be an immediate cause for concern due to a lack of other viable treatment options

Artemisinins are well-tolerated, offer a good safety profile, and rapid reduction in parasites. However, they also have fast clearance - so an artemisinin monotherapy treatment requires an extended treatment-period. Non-compliance by patients could lead to treatment failures and the development of resistance. ACTs are effective because they combine artemisinin with a slow-eliminating anti-malarial and are curative with only 3 days of treatment [102].

Artemisinin resistance is defined by a delay in parasite clearance time (the time from starting treatment until no parasites can be detected) with a slope-half-life greater than 5 hours [65, 115]. Delays in parasite clearance times have also been associated with host immunity and erythrocyte/hemoglobin polymorphisms [1, 3, 19, 26, 29, 74, 88].

The use of the slope half life allows an assessment of the clearance time which is independent of the baseline count [27, 105]. In addition, the standardized Parasite Clearance Estimator (PCE) software offered through WWARN, allows a standardized approach for measuring parasite clearance time and the slope half life which will facilitate comparisons with other studies and with data over time [71, 81, 119].

The aim of this research is to retrospectively analyze the data from a large multi-center study in SSA to explore if there are cases which meet the definition of artemisinin resistance and to characterize risk factors for such cases.

Importantly, this study occurred prior to the large scale implementation of ACT therapy in Africa, providing insight into the nature of "artemisnin resistance" prior to drug implementation.

2. Methods

The clinical data from this trial has been published separately and contains a more comprehensive description of the conduct of the individual trial, objectives and results from the original study design [70].

2.1. Objectives

The current analysis seeks to explore the parasite count data from the original clinical trial using the recently available Parasite Clearance Estimator (PCE) from WWARN - to provide consistent methodology for calculating the parasite clearance time and associated slope half life. This measure is used to assess the presence of artemisinin resistance and to explore the factors associated with impact of host immunity.

2.2. Participants

The initial study was conducted between June 2006 and August 2007 in five countries and 11 sites in sub-Saharan Africa: Burkina-Faso (1 center: Bobo-Dioulasso), Ghana (1 center: Kintampo), Kenya (3 centers: Eldoret, Kilifi, and Pingilikani), Nigeria (4 centers: Ibadan, Enugu,

Jos, and Calabar), and Tanzania (2 centers: Bagamoyo and Kiwangwa). Patients were treated with one of two artemisinin-combination therapies: chlorproguanil-dapsone-artesunate (CDA) or artemether-lumefantrine (AL). Both therapies utilized a 4mg dose of artemisinin.

Patients eligible for the trial had: uncomplicated *P.falciparum* malaria infection of 2,000 – 200,000 parasites/uL; were aged 12 months to 14 years old; and did not have co-morbidities or co-infection. Further details of the study entry criteria are included in the original publication [70].

2.3. Procedures

Baseline assessments included: temperature, age, height (or length); prior and current medical history; current medications; history of previous malaria episodes; and a baseline sample for assessment of G6PD status and hematologic and clinical chemistry laboratory data.

Patients were treated in-clinic for 3 days. A home visit occurred on days 4, 5, and 6. Then in-clinic follow-up visits occurred on days 7, 14, 28 and 42. At each clinic visit, samples were taken to assess parasite type and counts and to perform laboratory evaluations for clinical chemistry and hematology. Assessments for adverse events occurred at each visit and also were updated if reports were made between visits.

Parasite assessment

Asexual parasite and gametocyte counts were performed at screening, at pre-dose, every 8 hours during the in-patient stay until discharge on Day 3 and at follow-up visits on Days 7, 14, 28 and 42. At each time point, two thick and one thin film were prepared and parasite densities determined by examination of a thick blood slide (10 ML thumb prick), according to WHO methods. Two microscopists (blinded to treatment) examined each of the thick blood slides; and if there was a discrepancy a third reader also read the slides.

Parasite clearance time was assessed as the time needed to clear asexual parasite forms from the blood; with parasite numbers falling below the limit of detection in a thick blood smear and remaining undetectable for at least 48 hours.

2.4. Statistical methods

The slope half-life was calculated using the WWARN Parasite Clearance Estimator (PCE) to provide standardized calculations [119].

SAS version 9.3 was used for all analyses [78]. Descriptive statistics were used to report demographic and clinical characteristics for patients grouped by slope half life <= 5 hours and >5 hours. Categorical data is summarized as frequency and percent with p-values based on the chi-square test. Continuous data is presented with summary statistics: mean (standard deviation), median, minimum and maximum, with p-values based on analysis of variance (ANOVA). P-values are provided for descriptive purposes.

All statistical analyses were performed post-hoc. No formal sample size calculations were considered for the analyses reported in this paper. The sample size was planned based on the original clinical trial objectives and reported previously [70].

Correlations (using Spearman non-parametric correlations) among risk factors; and among risk factors and outcome were explored for continuous variables.

A logistic model was developed to investigate the influence of the following surrogates for host immunity (age, seasonality, estimated endemicity, geographic location – country and study site, and occurrence of previous malaria episodes), treatment and baseline parasitemia on parasite clearance time assessed as slope half life <=5 hours and > 5 hours.

Age category was assessed as <5 years or >= 5 years. Estimated endemicity was assessed using maps from the Malaria Atlas Project [47] with coordinates based on the center locations. Estimated endemicity is defined as the estimated proportion of 2-10 year olds in the general

population that are infected with *P. falciparum* at any one time - averaged over the 12 months of 2010 (also referenced as *Pf*PR – *P. falciparum* Parasite Rate). Estimated endemicity was explored as a continuous variable and categorized as <60% vs. >=60% [35]. Seasonality was assessed as 'Rainy' or 'Dry' based on the start date of the treatment and whether this corresponded to the rainy season for the geographic location of the center. The number of previous episodes was explored based on total number of episodes in the past 12 months and as a categorical variable: ever experiencing a previous episode (yes or no). Country-level and site-level investigations were also conducted. For modeling purposes, small sites were combined with sites in close geographic proximity.

Univariate logistic regression analyses were conducted to assess the influence of each individual parameter. A multivariate logistic regression analysis was also conducted which included the full set of candidate variables with a stepwise approach using a generous selection criterion of 0.20 for entry and 0.25 to retain the variable in the model.

A large number of patient profiles were excluded from the original analysis based on the threshold limits of the online WWARN PCE tool - in particular the requirement for the last detectable parasite count to be <1000. An additional sensitivity analysis was undertaken to assess these subjects by utilizing a higher threshold cut-off value of 10,000 allowing a slope half-life to be calculated for these subjects.

3. Results

3.1. Patients

A summary of the patient population available for analysis is shown in Figure 4.1. 1372 malaria patients were enrolled and all data was included for analysis in the PCE tool. Only approximately 80% of the subjects were analyzed by the PCE tool (1079 of the 1372 subjects). Of the 20% not analyzed – 9% (135/1372) were excluded due to too few data points; 1% (10/1372

subjects) were excluded due to parasitemia too low at baseline; 10% (148/1372 subjects) were excluded due to last positive parasitemia value exceeding 1,000.

Table 4.1 shows the demography summary for the analysis set (N=1079). Patients had a mean age of 4.2 years with a range of 1 to 14 years with 65% of subjects <5 years of age and 35% of subjects 5-15 years. Gender was similarly represented with 48% female and 52% male subjects.

Subjects were represented in 5 countries and 11 sites with the majority of subjects in Nigeria (42%, n=457), then Kenya (22%, n=239), Tanzania (20%, n=212), Ghana (15%, n=166) and Burkina Faso (<1%, n=5). Subjects were more commonly enrolled during the Rainy season (65%) than the Dry season (35%); and from moderate to highly endemic regions (Median: 50%, Range: 30%-60%).

Entry criteria for the study required subjects to have uncomplicated malaria with baseline parasitemia: 2,000 – 200,000 parasites/uL. Median baseline parasite count (ln) was 10.2, with range 7.0-13.5.

3.2. Parasite Clearance

Table 4.2 shows the summary statistics for the parasite clearance time for the set of patients analyzed with the PCE tool (N=1079). The median slope half life was 2.26 hours with range 1.16 to 28.03 hours; and inter-quartile range (25^{th} and 75^{th} percentiles) 1.84 to 2.76 hours. Twenty-four subjects (2%, 24 of the 1079 analyzed) had a slope half life >5 hours (13 occurring in 5-6 hours; 9 occurring in 6-12 hours; 1 occurring at 20-21 hours; and 1 occurring at 28-29 hours). Figure 4.2 shows the histogram for the slope half-life for the study.

3.3. Risk Factors for Delayed Parasite Clearance

Table 4.3 displays the demographic characteristics for subjects with parasite clearance slope half-life <=5 hours (N=1055) and the subjects with slow clearing parasites with slope half

life >5 hours (N=24). Figure 4.3 shows a graphical representation of the percentage of subjects with slope half life >5, by risk factor.

When comparing the demographic characteristics of subjects with slope half life <= 5 hours to those with slope half life >5 hours, we noted some differences to explore – though caution will be needed in the interpretation due to the small number of subjects with slope half life >5 hours. With only 24 patients, one patient moving to a different category could change the percent displayed by approximately 4%.

Patients with slow clearing parasites (slope half life >5 hours) tended to be older: 46% were age 5-<15 years compared to 35% for those with slope half life <=5 hours (p=0.275)

When assessing the location (country/site) where patients originated, we did observe an unusually high number of patients with slower clearing parasites coming from Nigeria, 79% (19 of 24 patients, p=0.003) and in particular from the Calabar site in Nigeria, 54% (13 of 24 patients). To explore further whether patients in this location possibly had a different baseline disease state - the baseline parasitemia was assessed by country and by site as shown in Figure 4.4. There were no apparent differences in the baseline parasitemia for Nigeria vs. the other countries and in particular for the Calabar site vs. the other sites.

Patients with slower clearing parasites were more likely to be female - 58% with slope half life >5 hours vs. 48% of patients with slope half life <=5 hours (p=0.328).

Patients with slower clearing parasites were more likely to have been treated during the rainy season than the dry season. Seventy-nine percent with slow clearing parasites were treated during the rainy season compared to 64% of the main group with slope half life <= 5 hours (p=0.131).

Patients with slower clearing parasites tended to be from areas with higher estimated endemicity (>= 60%). Fifty-four percent, 13 of the 24 patients with slow clearing parasites came from locations with estimated endemicity assessed as >=60% ($p<0.001$).

When assessing the impact of previous malaria episodes on slope half life, patients with slow clearing parasites were more likely to have reported a previous malaria episode (71%) than subjects with faster clearing parasites (56%) ($p=0.196$). A clear trend based on the number of episodes however was not apparent.

When assessing the impact of treatment on slope half life and realizing the original randomization scheme randomized CDA to AL in a 2:1 ratio - both treatment arms appeared to have similar representation for the group with slope half life >5 hours: 2% in the CDA group and only slightly higher 2.6% in the AL group ($p=0.571$).

Patients with slower clearing parasites also tended to have lower baseline parasitemia. Baseline parasitemia (ln) for subjects with slow clearing parasites was mean(sd),median 9.0(1.36),8.5 compared to 10.1(1.31),10.2 for subjects with faster clearing parasites ($p<0.001$). When analyzing the baseline parasitemia by quartile - the majority of the patients with slow clearing parasites had low baseline parasitemia: 58% of subjects were in Quartile 1; 29% in Quartile 2; 0 in Quartile 3; and 13% in Quartile 4; compared to approximately 25% in each Quartile for subjects with faster clearing parasites.

The sensitivity analysis included slope half life data for 146 additional subjects who had a parasite count above the standard PCE threshold followed by an undetectable assessment(increasing the total number of subjects for analysis from 1079 to 1225). This included 144 patients with slope half-life <5 hours and 2 additional patients with a slope half-life >5 hours - increasing this count from 24 to 26 patients. The results from the sensitivity analysis were consistent with the above results and are not shown separately.

3.4. Correlates and Risk Factors for Delayed Parasite Clearance

A logistic regression analysis was conducted to investigate the influence of risk factors such as baseline parasitemia, treatment group, sex and surrogates for host immunity (age, seasonality, estimated endemicity, geographic location and number of previous malaria episodes) on parasite clearance time assessed as slope half life <=5 hours and > 5 hours. In the univariate analysis - country, site, baseline parasitemia and estimated endemicity each revealed a significant influence or association with slope half life (Table 4.4). Of note, when modeling site, due to the many sites (n=11) and the smaller centers - many geographic areas were combined to allow for a meaningful analysis. Only 5 subjects were enrolled in Burkina Faso. These subjects were combined with subjects from the neighboring country of Ghana for analysis. To allow estimation for Kenya and Tanzania which had small centers - all centers within each country were combined for analysis.

In the multivariate analysis shown on Table 4.5, site ($p<0.001$) and baseline parasitemia ($p<0.001$) remained important factors influencing delayed parasite clearance. Sex ($p=0.112$) also met the criteria for selection and retention in the model but only appeared to have a marginal contribution. Estimated endemicity and country did not provide any important additional information once site entered the model and were not included in the final model.

For site, reference coding was used to compare each site to Tanzania for descriptive information. Odds ratios (OR) and 95% Confidence intervals (CI) are provided. As expected, the Calabar site in Nigeria was dramatically different with OR 8.64 (95% CI 1.87, 39.96). Baseline parasitemia, when controlling for site and sex, also had a strong association with delayed parasite clearance with an OR 0.46 (95% CI 0.31, 0.69).

4. Discussion

The primary objective of this research was to explore potential risk factors for slowed parasite clearance time and investigate whether increased PCT could be due to naturally occurring variation.

By definition, artemisinin resistance is slowed parasite clearance time assessed as slope half life greater than 5 hours. Analysis of the data from this study identified several cases with slowed clearance time meeting this definition. However, the data collection for this study occurred in 2006-2007 which pre-dates the introduction of widespread use of ACTs in this region. Therefore, finding delayed PCT in this study likely does not indicate ACT induced resistance, but possibly a naturally occurring variation or natural selection of resistant variants [116].

The analysis identified geographic location or site as one of the most important risk factors. One site in particular contributed a disproportionate number of subjects with delayed parasite clearance (54%, 13 of the 24 patients with slope half life >5 hours). Analysis of the baseline parasitemia at this site was similar to other sites and the overall population and did not indicate this site had patients with higher parasitemia or a sicker population. This site, along the southern coast-line of Nigeria near the Cameroon border, has a longer rainy season and a higher estimated endemicity (>=60%) than other sites. However, it is likely that this resistant phenotype was due to natural genetic variation.

The current study offers relevant results for assessing clinical determinants for delayed parasite clearance time for malaria treatment of subjects in SSA - some limitations of this research, however, are described below.

Mutations in the K13-propeller gene have been found to strongly correlate with delayed parasite clearance time in parts of Asia (Ariey, 2014; Mohon, 2014; Plowe, 2014). Mutations in this gene have also been found in SSA (Conrad, 2014; Kamau, 2014; and Taylor, 2014).

Unfortunately, parasite samples from our study were no longer available, so we could not look for the presence or absence of these mutations. Similarly, the recent findings of delayed parasite clearance associated with hemoglobin type (Amaratunga, 2012; Fairhurst, 2012) could not be explored as this data was not collected in this study.

The study population is limited to the specific centers and countries studied and two particular ACTs, and the results are limited to the data and covariates collected and analyzed in this study. The inclusion of additional factors might provide different results in combination with the factors presented and a different overall model.

The study entry criteria did not allow patients with other underlying disease; co-infections; known G6PD deficiency, or severe/complicated malaria to be entered in the study. Therefore the generalizability of the study is limited to the characteristics of the inclusion criteria.

Also of note, some of the derived data such as seasonality and estimated endemicity may be considered 'soft' data and while it is helpful for exploratory research would require a more directed and precise assessment for future investigative analyses. Estimated endemicity data was based on available maps from 2010, which may have differed from endemicity data in 2007 had this been available. Also, the estimated endemicity data is derived from maps produced with a modeling approach to assess endemicity across regions and was not intended to provide point estimates for specific locations. Similarly, seasonality was based on typical months of the rainy and dry seasons for each country from travel data and maps available from Mapping Malaria Risk in Africa [48] and again is providing a general assessment and not intended to provide accurate rainfall amounts for precise locations. In addition, the start of a rainy season may not indicate the actual start of intense malaria transmission – which may be days or weeks into the rainy season.

Both of these variables were also based on geographic location or site to make these assessments and therefore a correlation with site is expected when analyzing this data.

Slope half life was calculated using the standardized PCE tool available from WWARN. This does provide a consistent approach for assessing slope half life across malaria studies and is an important advancement in this area. However, some of the thresholds in the online program may be too restrictive, eliminating large amounts of real data which may provide useful information for analysis. For this study, 10-20% of the data was originally eliminated from analysis due to these stringent thresholds. The sensitivity analysis including this data did not indicate different conclusions would have been reached for this particular analysis. However, it may be useful to assess the impact these threshold values may have on other datasets and research as this PCE tool becomes more widely used.

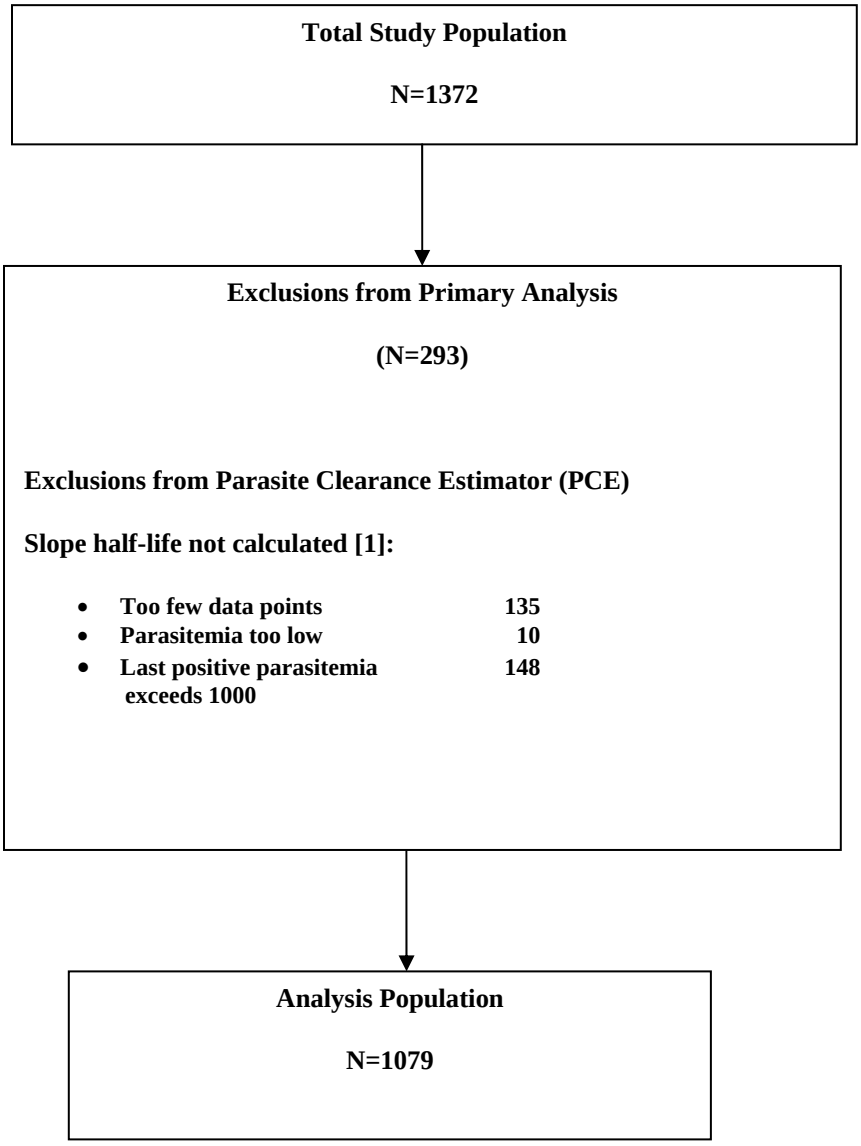
5. Conclusions

This analysis utilizing data from a large well-controlled clinical trial in SSA, yields a large dataset with which to explore determinants that would not be possible with the smaller datasets or case-studies currently available. The data collected for this study prior to the widespread use of ACTs in this region offered a unique opportunity to uncover the occurrence of delayed PCT which would not be tied to therapy-induced resistance, but may be part of a natural selection process inherent in geographic regions.

Some delayed PCT pre-existed the introduction of artemisinin combinations in Africa. This suggests that resistant parasite strains, particularly in some parts of Africa, pre-existed the introduction of ACTs.

6. Tables and Figures

Figure 4.1. Consort Diagram



[1] Analyses using WWARN Parasite Clearance Estimator (PCE) available online.

Figure 4.2. Distribution of Slope Half Life

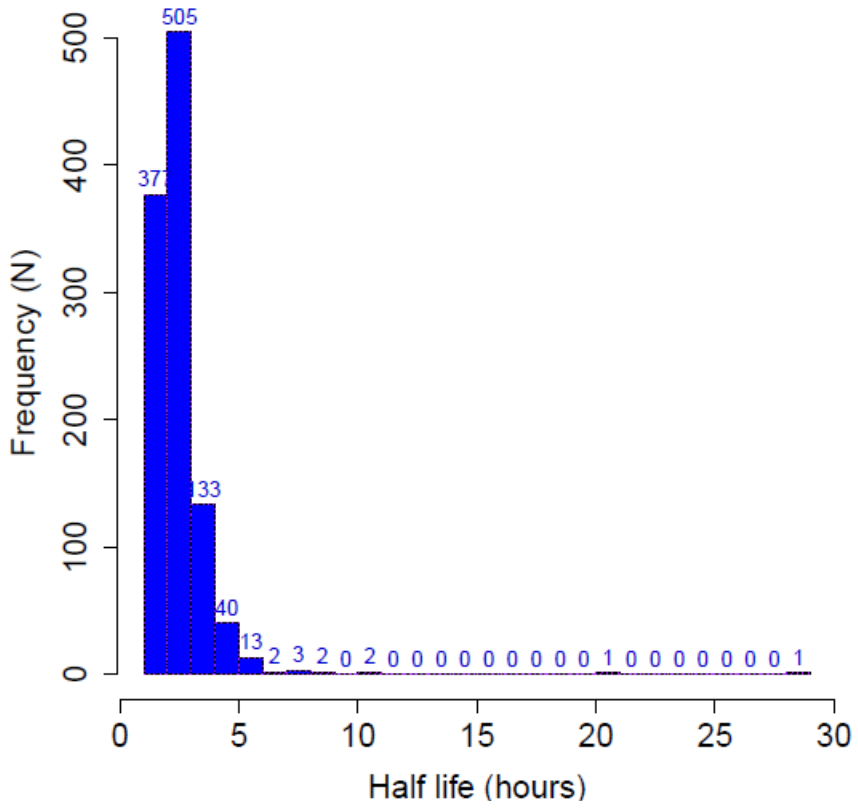
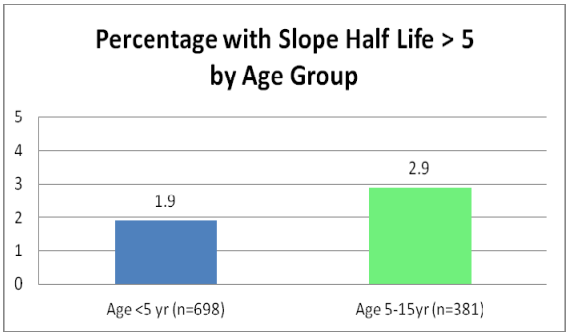
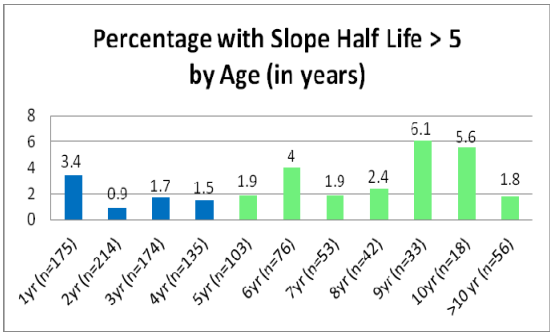
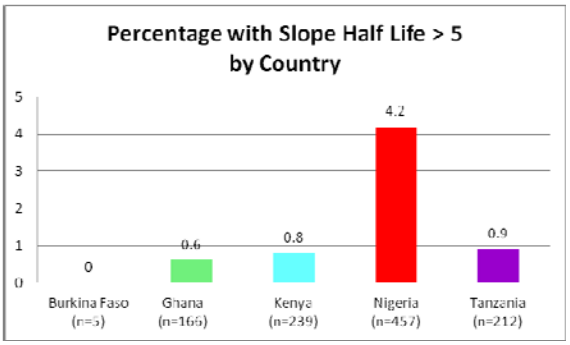


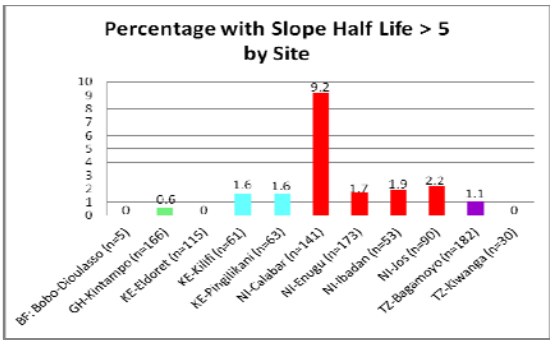
Figure provided from WWARN Parasite Clearance Estimator Report.

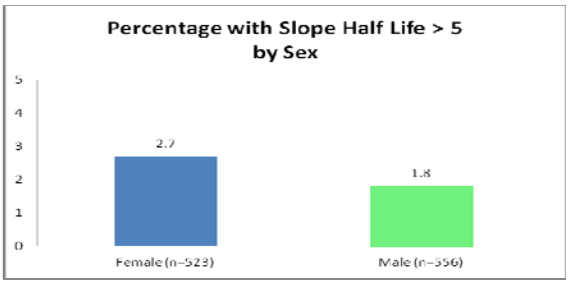
Figure 4.3. Percentage of Subjects with Slope Half Life >5, by Risk Factor

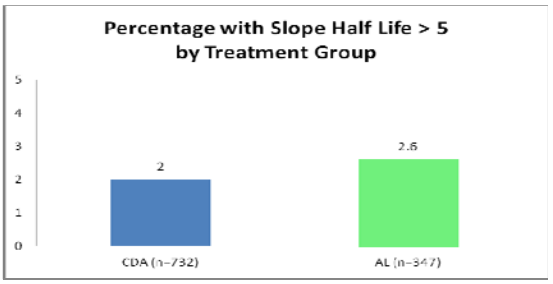


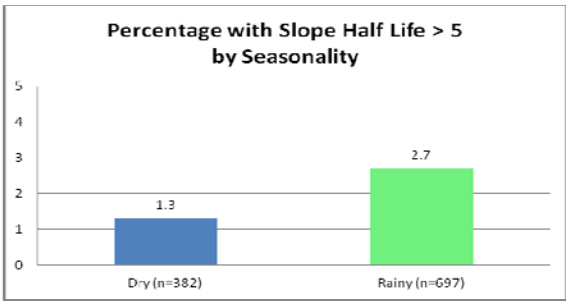


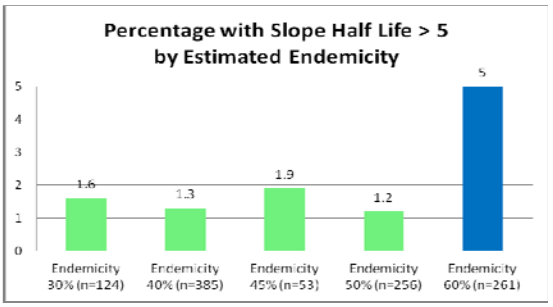


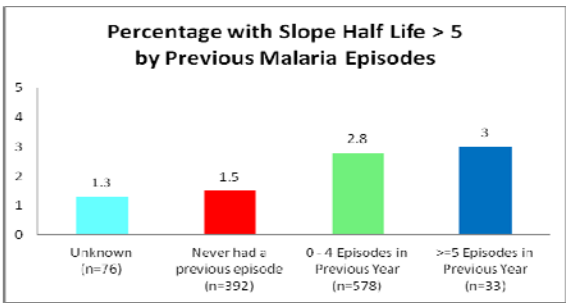












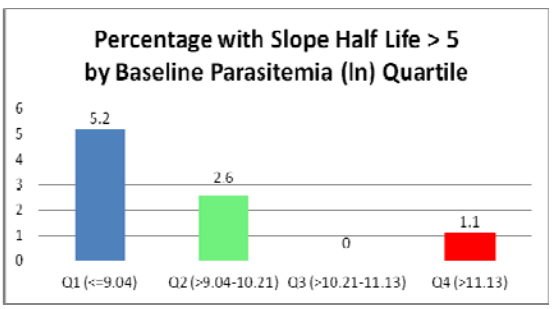
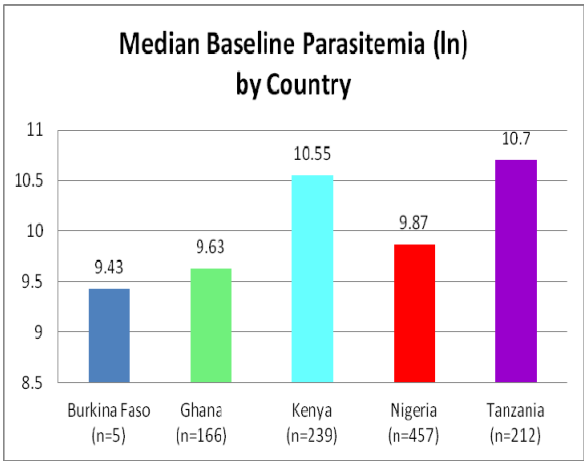
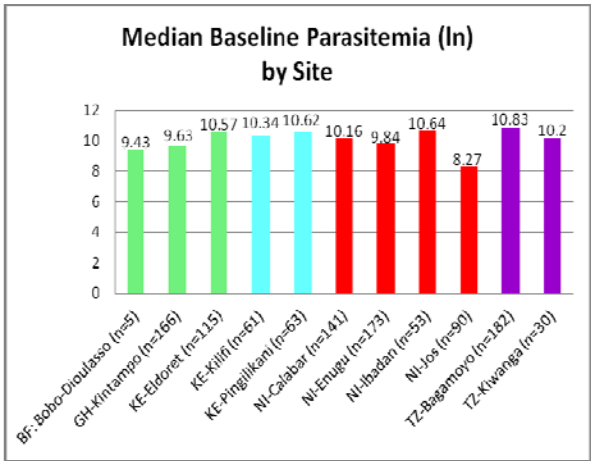
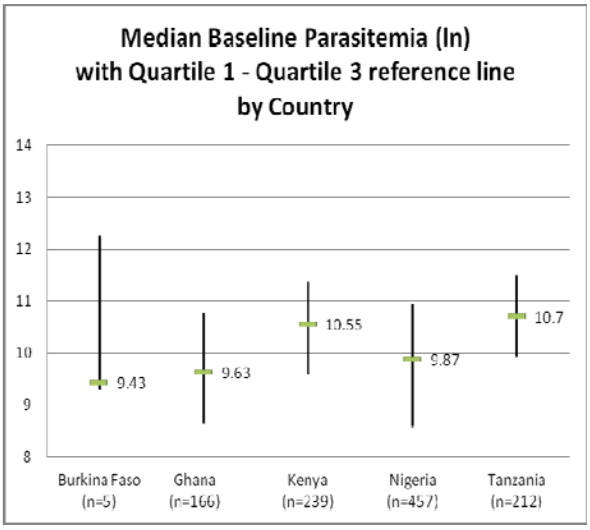


Figure 4.4. Baseline Parasitemia (ln), by Country and Site







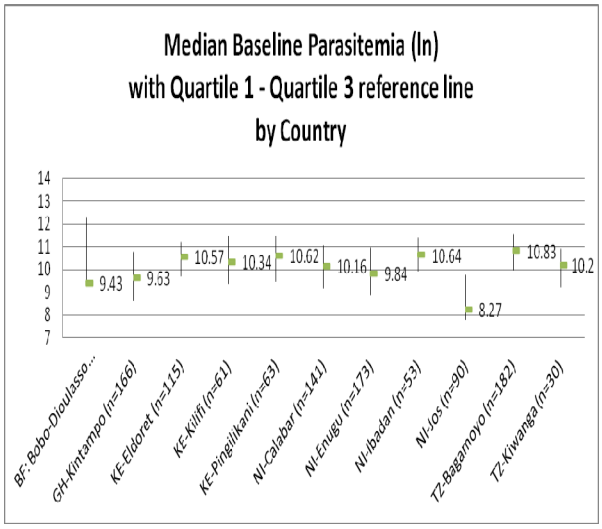


Table 4.1. Baseline Characteristics

	Total (n=1079)
Age (years)	
Mean (sd)	**4.2 (2.90)**
Median	**3.0**
Min – Max	**1-14**
Age Group	
<5 years	**698 (65%)**
5 to 15 years	**381 (35%)**
Sex	
Female	**523 (48%)**
Male	**556 (52%)**
Country	
Burkina Faso	**5 (<1%)**
Ghana	**166 (15%)**
Kenya	**239 (22%)**
Nigeria	**457 (42%)**
Tanzania	**212 (20%)**
Seasonality	
Dry	**382 (35%)**
Rainy	**697 (65%)**
Estimated Endemicity	
Mean (sd)	**0.5 (0.10)**
Median	**0.5**
Min – Max	**0.3 – 0.6**
Previous Malaria Episode	
Yes	**611 (57%)**
No	**392 (36%)**
Unknown	**75 (7%)**
Baseline Parasite Count	
Mean (sd)	**48,727 (58,026.3)**
Median	**27,136**
Min – Max	**1,068-705,600**
Baseline Parasite Count (ln)	
Mean (sd)	**10.1 (1.32)**
Median	**10.2**
Min – Max	**7.0-13.5**

Table 4.2. Summary Statistics for Parasite Clearance

Summary of clearance rate constant (/hour)

Statistic	value
Median	0.31
Range*	(0.02,0.60)
IQR**	(0.25,0.38)

*Range = (minimum, maximum)

**IQR = Inter Quartile Range; (25th centile, 75th centile)

Summary of slope half life

Statistic	value
Median	2.26
Range*	(1.16,28.03)
IQR**	(1.84,2.76)

*Range = (minimum, maximum)
**IQR = Inter Quartile Range; (25th centile, 75th centile)

Slope half life distribution

Slope half life (hours)*	N	%	Cumulative %
0 to 2	377	34.94	34.94
2 to 3	505	46.80	81.74
3 to 4	133	12.33	94.07
4 to 5	40	3.71	97.78
5 to 6	13	1.20	98.98
6 to 7	2	0.19	99.17
7 to 8	3	0.28	99.44
8 to 9	2	0.19	99.63
9 to 10	0	0.00	99.63
10 to 11	2	0.19	99.81
11 to 12	0	0.00	99.81
12 to 13	0	0.00	99.81
13 to 14	0	0.00	99.81
14 to 15	0	0.00	99.81
15 to 16	0	0.00	99.81
16 to 17	0	0.00	99.81
17 to 18	0	0.00	99.81
18 to 19	0	0.00	99.81
19 to 20	0	0.00	99.81
20 to 21	1	0.09	99.91
21 to 22	0	0.00	99.91
22 to 23	0	0.00	99.91
23 to 24	0	0.00	99.91
24 to 25	0	0.00	99.91
25 to 26	0	0.00	99.91
26 to 27	0	0.00	99.91
27 to 28	0	0.00	99.91
28 to 29	1	0.09	100.00

*each interval includes the left-hand value and excludes the right-hand value

Summaries provided from WWARN Parasite Clearance Estimator Report.

Table 4.2. Summary Statistics for Parasite Clearance (continued)

	Total (n=1079)
Parasite Clearance Time (hours)	
Mean (sd)	**26.0 (10.47)**
Median	**24.0**
Min – Max	**8.0-72.0**
Parasite Clearance Time (hours)	
<= 8 hours	**1 (<1%)**
>8–16 hours	**367 (34%)**
>16–24 hours	**329 (30%)**
>24-32 hours	**230 (21%)**
>32-40 hours	**83 (8%)**
>40-48 hours	**46 (4%)**
>48-56 hours	**9 (<1%)**
>56-64 hours	**6 (<1%)**
>64-72 hours	**5 (<1%)**
>72 hours	**3 (<1%)**

Table 4.3. Risk Factors for Delayed Parasite Clearance

	Slope half life 0-5 (n=1055)	Slope half life >5 (n=24)	Row Percent[1]
Age (years)			
Mean (sd)	**4.1 (2.89)**	**4.5 (3.18)**	
Median	**3.0**	**4.0**	
Min – Max	**1-14**	**1-11**	
Age Group			
<5 years	**685 (65%)**	**13 (54%)**	**2%**
5 to 15 years	**370 (35%)**	**11 (46%)**	**3%**
Age (years)			
1 year	**169 (16%)**	**6 (25%)**	**3%**
2 years	**212 (20%)**	**2 (8%)**	**1%**
3 years	**171 (16%)**	**3 (13%)**	**2%**
4 years	**133 (13%)**	**2 (8%)**	**2%**
5 years	**101 (10%)**	**2 (8%)**	**2%**
6 years	**73 (7%)**	**3 (13%)**	**4%**
7 years	**52 (5%)**	**1 (4%)**	**2%**
8 years	**41 (4%)**	**1 (4%)**	**2%**
9 years	**31 (3%)**	**2 (8%)**	**6%**
>=10 years	**72 (7%)**	**2 (8%)**	**3%**
Country			
Burkina Faso	**5 (<1%)**	**0**	**0**
Ghana	**165 (16%)**	**1 (4%)**	**1%**
Kenya	**237 (22%)**	**2 (8%)**	**1%**
Nigeria	**438 (42%)**	**19 (79%)**	**4%**
Tanzania	**210 (20%)**	**2 (8%)**	**1%**
Site			
Burkina Faso: Bobo-Dioulasso	**5 (<1%)**	**0**	**0**
Ghana: Kintampo	**165 (16%)**	**1 (4%)**	**1%**
Kenya: Eldoret	**115 (11%)**	**0**	**0**
Kenya: Kilifi	**60 (6%)**	**1 (4%)**	**2%**
Kenya: Pingilikani	**62 (6%)**	**1 (4%)**	**2%**
Nigeria: Calabar	**128 (12%)**	**13 (54%)**	**9%**
Nigeria: Enugu	**170 (16%)**	**3 (13%)**	**2%**
Nigeria: Ibadan	**52 (5%)**	**1 (4%)**	**2%**
Nigeria: Jos	**88 (8%)**	**2 (8%)**	**2%**
Tanzania: Bagamoyo	**180 (17%)**	**2 (8%)**	**1%**
Tanzania: Kiwangwa	**30 (3%)**	**0**	**0**

Table 4.3. Risk Factors for Delayed Parasite Clearance (continued)

	Slope half life 0-5 (n=1055)	Slope half life >5 (n=24)	Row Percent[1]
Sex			
Female	**509 (48%)**	**14 (58%)**	**3%**
Male	**546 (52%)**	**10 (42%)**	**2%**
Treatment Group			
CDA	**717 (68%)**	**15 (63%)**	**2%**
AL	**338 (32%)**	**9 (38%)**	**3%**
Seasonality			
Dry	**377 (36%)**	**5 (21%)**	**1%**
Rainy	**678 (64%)**	**19 (79%)**	**3%**
Estimated Endemicity			
Mean (sd)	**0.5 (0.10)**	**0.5 (0.11)**	
Median	**0.5**	**0.6**	
Min – Max	**0.3 – 0.6**	**0.3 – 0.6**	
Estimated Endemicity (%)			
30%	**122 (12%)**	**2 (8%)**	**2%**
40%	**380 (36%)**	**5 (21%)**	**1%**
45%	**52 (5%)**	**1 (4%)**	**2%**
50%	**253 (24%)**	**3 (13%)**	**1%**
60%	**248 (24%)**	**13 (54%)**	**5%**
Previous Malaria Episode			
Yes	**594 (56%)**	**17 (71%)**	**3%**
No	**386 (37%)**	**6 (25%)**	**2%**
Unknown	**74 (7%)**	**1 (4%)**	**1%**
Episodes in Previous Year			
0	**40 (7%)**	**2 (12%)**	**5%**
1	**240 (40%)**	**5 (29%)**	**2%**
2	**146 (25%)**	**5 (29%)**	**3%**
3	**105 (18%)**	**2 (12%)**	**2%**
4	**31 (5%)**	**2 (12%)**	**6%**
5	**17 (3%)**	**1 (6%)**	**6%**
>5	**15 (3%)**	**0**	**0**

Table 4.3. Risk Factors for Delayed Parasite Clearance (continued)

	Slope half life 0-5 (n=1055)	Slope half life >5 (n=24)	Row Percent[1]
Baseline Parasite Count			
Mean (sd)	**49,322 (58,209.8)**	**22,576 (42,501.0)**	
Median	**27,768**	**5,096**	
Min – Max	**1,068-705,600**	**1,626-175,786**	
Baseline Parasite Count (ln)			
Mean (sd)	**10.1 (1.31)**	**9.0 (1.36)**	
Median	**10.2**	**8.5**	
Min-Max	**7.0-13.5**	**7.4-12.1**	
Baseline Parasite Count (ln) Quartiles			
Quartile 1 (<= 9.04)	**256 (24%)**	**14 (58%)**	**5%**
Quartile 2 (> 9.04 – 10.21)	**263 (25%)**	**7 (29%)**	**3%**
Quartile 3 (> 10.21 – 11.13)	**269 (26%)**	**0**	**0**
Quartile 4 (> 11.13)	**267 (25%)**	**3 (13%)**	**1%**
Baseline Parasite Count (ln)			
<= Median (<=10.21)	**519 (49%)**	**21 (88%)**	**4%**
>Median (>10.21)	**536 (51%)**	**3 (13%)**	**1%**
Parasite Clearance Time (hours)			
Mean (sd)	**25.5 (9.72)**	**47.8 (17.36)**	
Median	**24.0**	**47.5**	
Min – Max	**8.0-72.0**	**16.0-72.0**	
Parasite Clearance Time (hours)			
<= 8 hours	**1 (<1%)**	**0**	**0**
>8–16 hours	**366 (35%)**	**1 (4%)**	**<1%**
>16–24 hours	**327 (31%)**	**2 (8%)**	**1%**
>24-32 hours	**226 (21%)**	**4 (17%)**	**2%**
>32-40 hours	**80 (8%)**	**3 (13%)**	**4%**
>40-48 hours	**41 (4%)**	**5 (21%)**	**11%**
>48-56 hours	**7 (<1%)**	**2 (8%)**	**22%**
>56-64 hours	**4 (<1%)**	**2 (8%)**	**33%**
>64-72 hours	**2 (<1%)**	**3 (13%)**	**60%**
>72 hours	**1 (<1%)**	**2 (8%)**	**67%**

[1] Row percent is the percent of subjects with slope half life >5 hours for each sub-category.

Table 4.4. Logistic Regression Analysis - Univariate Analysis

Parameter(s)	Odds Ratio (95% CI)	p-value
Age Group (<5 vs. 5-15 years)	0.64 (0.28, 1.44)	0.279
Sex (Female vs. Male)	1.50 (0.66, 3.41)	0.331
Estimated Endemicity (<60% vs. >=60%)	0.26 (0.12, 0.59)	0.001
Seasonality (Dry vs. Rainy)	0.47 (0.18, 1.28)	0.140
Previous Malaria Episodes (No vs. Yes)	0.54 (0.21, 1.39)	0.203
Country		0.012
Country(Burkina Faso/Ghana vs. Tanzania)	0.62(0.06, 6.87)	
Country(Kenya vs. Tanzania)	0.89 (0.12, 6.35)	
Country(Nigeria vs. Tanzania)	4.56 (1.05, 19.74)	
Site		<0.001
Site (Burkina Faso/Ghana vs. Tanzania)	0.62(0.06, 6.87)	
Site (Kenya vs. Tanzania)	0.89 (0.12, 6.35)	
Site (Nigeria: Calabar vs. Tanzania)	10.66 (2.37, 48.03)	
Site (Nigeria: Enugu vs. Tanzania)	1.85 (0.31, 11.22)	
Site (Nigeria: Ibadan vs. Tanzania)	2.02 (0.18, 22.70)	
Site (Nigeria: Jos vs. Tanzania)	2.39 (0.33, 17.21)	
Baseline Parasite Count(ln)	0.50 (0.36, 0.71)	<0.001
Treatment Group (CDA vs. AL)	0.79 (0.34, 1.81)	0.572

Outcome: Slope half life (0-5, >5hours)
Modeling probability slope half life >5 hours

Table 4.5. Logistic Regression Analysis - Multivariate Analysis

Parameter(s)	Odds Ratio (95% CI)	p-value
Site		**<0.001**
Site (Burkina Faso/Ghana vs. Tanzania)	**0.41 (0.04, 4.66)**	
Site (Kenya vs. Tanzania)	**0.43 (0.04, 4.81)**	
Site (Nigeria: Calabar vs. Tanzania)	**8.64 (1.87, 39.96)**	
Site (Nigeria: Enugu vs. Tanzania)	**1.09 (0.17, 6.84)**	
Site (Nigeria: Ibadan vs. Tanzania)	**4.18 (0.35, 50.13)**	
Site (Nigeria: Jos vs. Tanzania)	**0.73 (0.09, 5.75)**	
Baseline Parasite Count (ln)	**0.46 (0.31, 0.69)**	**<0.001**
Sex (Female vs. Male)	**2.04(0.84, 4.96)**	**0.117**

Outcome: Slope half life (0-5, >5hours)
Modeling probability slope half life >5 hours

CHAPTER 5: RISK FACTORS FOR RECRUDESCENCE

1. Introduction

Artemisinin containing therapies (ACTs) are currently the first-line treatment recommended by the World Health Organization (WHO) for the treatment of uncomplicated *Plasmodium falciparum* in sub-Saharan Africa (SSA). The efficacy with these treatments is often >=95%. The artemisinin component is also well-tolerated, offers a good safety profile, and rapid reduction in parasites; and importantly reduces the risk of resistance – which has caused many previous treatments to become ineffective [67, 102].

Treatment failure with ACTs however does still occur and it is important to explore what factors may contribute to this occurrence and if the risk factors may be "host-related" - possibly indicating changes in immunity; or "parasite-related" - possibly indicating signs of resistance.

In order to assess treatment failure it is also important to be able to distinguish which recurrences or return of parasitemia are due to true failure or "recrudescence" (return of the original infection) and which cases may be the result of a different newly acquired infection or "new infection" - which would not be considered a failure. PCR molecular genotyping is conducted to assess if the genetic make-up of the infection is consistent with the original infection assessed at baseline or contains new markers not previously seen. Initially only 2 regions were genotyped, but a more robust approach with three regions: *Pf*MSP-1, *Pf*MSP-2, and *Pf*GLURP is the WHO recommended approach to more accurately assess recrudescence and the approach used for these analyses [10, 87, 108]. In addition to identifying a recurrence as "recrudescence" or "new infection", there is also a third possibility which may be identified with the PCR analysis -

indicating the recurrence is a combination of a “recrudescence plus a new infection”. These cases would typically also be considered as a failure due to the observed recrudescence.

Previous research exploring risk factors for recrudescence has indicated baseline parasitemia and host factors associated with changes in immunity may influence recrudescence [19, 29, 38, 74, 80, 88].

Resistance can also contribute to treatment failure - and especially in the early onset of resistance in a geographic region it may be difficult to assess when this is actually occurring. Delayed parasite clearance time assessed as a slope half life >5 hours is the clinical definition of resistance [2, 3, 65, 115]. The inclusion of the slope half life (representing the time to clear 50% of initial parasites) as a potential risk factor may aid in assessment of an association between resistance and recrudescence. Parasite clearance measures including slope half life have been used as common assessments for malaria studies. However, the differences in how these measures have been calculated have lead to inconsistencies in reporting. The standardized Parasite Clearance Estimator (PCE) software recently offered through WWARN, allows a standardized approach for measuring parasite clearance time and the slope half life which will facilitate comparisons with other studies and with data over time and allow for the meaningful consistent application of definitions such as the recent definition for resistance [71]. The use of slope half life is also a preferred measure, as it removes the dependence on baseline parasitemia levels [27, 105, 119].

The aim of this research is to retrospectively analyze the data from a large multi-center study in SSA to explore the occurrence of recrudescence; and to further explore the risk factors associated with recrudescence to assess if they are likely host-related rather than parasite-related.

2. Methods

A comprehensive description of the conduct of the individual trial, objectives, inclusion and exclusion criteria, and results from the original study design has been previously published [70].

The current analysis seeks to further explore data from the original clinical trial to assess risk factors for recrudescence. Along with baseline parasitemia, treatment, and other host factors such as age, seasonality, estimated endemicity, geographic location and previous malaria episodes; slope half life data - using the recently available Parasite Clearance Estimator (PCE) from WWARN will also be explored.

2.1. Assessment of Recrudescence

Recrudescence is defined as a return of initial parasites either alone or in combination with a new infection. The determination of recrudescence status was based on the Polymerase Chain Reaction (PCR) data reported in the parent study which assessed genetic markers: merozoite surface proteins 1 and 2 (MSP-1, MSP-2) and glutamate-rich protein (GLURP) to identify parasites from the initial infection vs. parasites not identified previously and therefore indicative of a new infection. This assessment was made by comparing the baseline sample to the first positive sample on or after Day 7 when parasites reappeared.

2.2. Statistical methods

SAS version 9.3 was used for all analyses [78]. Descriptive statistics were used to report demographic and clinical characteristics for patients grouped as: No Recrudescence ("No return of parasitemia/No parasites detected" or a return of parasitemia as a "new infection only") and Recrudescence ("recrudescence only" or "recrudescence plus new infection").

Categorical data is summarized as frequency and percent with p-values based on the chi-square test. Continuous data is presented with summary statistics: mean (standard deviation),

median, minimum and maximum, with p-values based on analysis of variance (ANOVA). P-values are provided for descriptive purposes.

All statistical analyses were performed post-hoc. No formal sample size calculations were considered for the analyses reported in this paper. The sample size was planned based on the original clinical trial objectives and reported previously [70].

A logistic model was developed to investigate the influence of the following surrogates for host immunity (age, seasonality, estimated endemicity, geographic location – country and study site, and occurrence of previous malaria episodes), baseline parasitemia, treatment group, sex and slope half life on recrudescence.

Age category was assessed as <5 years or >= 5 years. The occurrence of previous malaria episodes was explored as a categorical variable: ever experiencing a previous episode (yes or no). Country-level and site-level investigations were also conducted. For modeling purposes, small sites were combined with sites in close geographic proximity.

Seasonality was assessed as 'Rainy' or 'Dry' based on the start date of the treatment and whether this corresponded to the rainy season for the geographic location of the center. Estimated endemicity was assessed using maps from the Malaria Atlas Project [47] with coordinates based on the center locations. Estimated endemicity is defined as the estimated proportion of 2-10 year olds in the general population that are infected with P. falciparum at any one time - averaged over the 12 months of 2010. Estimated endemicity was categorized as <60% vs. >=60%.

The slope half-life was calculated using the WWARN Parasite Clearance Estimator (PCE) to provide standardized calculations [119]. Summary statistics, summary of results by quartile and percentages above and below the median value are presented. A summary of results for subjects with slope half life <=5 hours and >5 hours are also presented to correspond to the clinical definition of resistance.

Univariate logistic regression analyses were conducted to assess the influence of each individual parameter. A multivariate logistic regression analysis was also conducted which included the full set of candidate variables with a stepwise approach using a generous selection criterion of 0.20 for entry and 0.25 to retain the variable in the model.

3. Results

3.1. Patients

A summary of the patient population available for analysis is shown in Figure 5.1. 1372 malaria patients were enrolled with 1293 subjects having data available for assessment for this analysis. Four percent (52 of 1293 subjects) had recrudescence during this study.

Table 5.1 shows the demography summary for the analysis set (N=1293). Patients had a mean age of 4.2 years with a range of 1 to 14 years with 65% of subjects <5 years of age and 35% of subjects 5-15 years. Gender was similarly represented with 49% female and 51% male subjects.

Subjects were represented in 5 countries and 11 sites with the majority of subjects in Nigeria (39%, n=504), then Tanzania (24%, n=308), Kenya (22%, n=290), Ghana (14%, n=186) and Burkina Faso (<1%, n=5). Subjects were more commonly enrolled during the Rainy season (62%) than the Dry season (38%); and from moderate to highly endemic regions (Median: 40%, Range: 30%-60%).

Median baseline parasite count (ln) was 10.2, with range 0-13.5.

3.2. Recurrence of Parasitemia

Table 5.2 shows the classification and timing of recurrent cases. Based on PCR assessment, 84% of recurrent cases were classified as “New Infection only” (n=278) and 16% were classified as “Recrudescent” (n=52) - which included cases with “recrudescence only” (31 of 52) and cases with a combination of “recrudescence plus new infection” (21 of 52).

Overall treatment compliance for the trial was previously reported as 94% (assessed as subjects receiving the correct treatment and dose on all 3 days). When looking at this data by recurrence status, non-compliance was higher in the "recrudescence only" group (13%, 4 of 31) and the "recrudescence plus new infection" group (10%, 2 of 21) than in the "new infection only" group (4%, 10 of 278) - although the number of subjects with noncompliance was low.

When assessing the slope half life data by recurrence status, a trend is noted with subjects in the "recrudescence only" group having longer slope half life (median 2.5), then subjects in the "recrudescence plus new infection" group (median 2.4) and subjects in the "new infection only" group had the shortest half life (median 2.2). The quartile data showed a more obvious trend with only 20% of recrudescent subjects having a slope half life below the median value and approximately 80% having slope half life greater than the median value. The slope half life for the new infection group was more evenly distributed across the quartiles. Very few subjects had a slope half life >5 hours, however, a higher percentage of these subjects were in the recrudescent group (7%, 2 of 29) than in the new infection group (2%, 6 of 263). Due to the small numbers, caution should be used with this interpretation.

The timing of the recurrence was similar for "recrudescent only" cases and "recrudescence plus new infection" cases, with about 15% of recurrence occurring within 14 days and the majority of cases (approximately 54%) occurring after day 14 and up to the day 28 assessment. The timing of recurrence was slightly delayed for the "new infection" group with only 4% of recurrence occurring within 14 days, and then the majority of cases (54%) also occurring after day 14 and up to the day 28 assessment.

3.3. Risk Factors for Recrudescence

Table 5.3 displays the demographic characteristics for subjects with "no recrudescence" (subjects with no return of parasitemia or a return of parasitemia determined to be a "new infection

only") (N=1241) and the subjects with "recrudescence" (subjects with "recrudescence only" or a combination of "recrudescence plus new infection") (N=52). Figure 5.2 shows a graphical representation of the percentage of subjects with recrudescence, by risk factor.

When comparing the demographic characteristics of subjects with recrudescence to those with no recrudescence, we noted some differences to explore – though some caution may be needed in the interpretation due to the small number of subjects with recrudescence.

Patients with recrudescence tended to be younger: 73% were age 5-<15 years compared to 64% for those without recrudescence (p=0.191).

When assessing the location (country/site) where patients originated, we did observe an unusually high number of patients with recrudescence coming from Nigeria, 50% with 26 of 52 patients (p=0.078) and in particular from the Calabar site in Nigeria, 25% (13 of 52 patients). To explore further whether patients in this location possibly had a different baseline disease state - the baseline parasitemia was assessed by country and by site. No apparent differences were observed in the baseline parasitemia for Nigeria vs. the other countries and in particular for the Calabar site vs. the other sites.

Patients with recrudescence were more likely to be female, 58% with recrudescence vs. 49% of patients without recrudescence (p=0.202).

Patients with recrudescence were more likely to have been treated during the rainy season than the dry season. Seventy-nine percent with recrudescence were treated during the rainy season compared to 62% without recrudescence (p=0.013).

Patients with recrudescence tended to be from areas with higher estimated endemicity (>= 60%). Thirty-seven percent, 19 of the 52 patients with recrudescence came from locations with estimated endemicity assessed as >=60%, compared to 21% for patients without recrudescence (p=0.009).

When assessing the impact of previous malaria episodes on recrudescence, patients with recrudescence were more likely to have reported a previous malaria episode (60%) than subjects without recrudescence (56%). Of note, 87 patients did not record information regarding the history of previous malaria episodes. This included thirteen percent (7 of 52) of the patients with recrudescence that were therefore not evaluable for this factor. When removing these subjects with missing data, 69% of subjects with recrudescence reported having a previous malaria episode compared with 60% of subjects without recrudescence (p=0.238). A clear trend based on the number of episodes however was not apparent.

When assessing the impact of treatment group on recrudescence, and realizing the original randomization scheme randomized CDA to AL in a 2:1 ratio - the CDA treatment group had a greater representation in the group with recrudescence: 5.2% (45 of 861) in the CDA group and only 1.6% (7 of 432) in the AL group (p=0.002).

Patients with recrudescence appeared to have similar baseline parasitemia. Median baseline parasitemia (ln) for patients with and without recrudescence were both 10.2. Mean(SD) for each group 10.1(1.43) and 10.2(1.29) were also similar (p=0.576). When analyzing the baseline parasitemia by quartile – patients with recrudescence were less likely to have low levels of parasitemia (within the first quartile). However, a strong trend across quartiles did not appear.

When assessing parasite clearance time and slope half life (time to clear 50% of parasites), the patients with recrudescence appeared to have a slightly longer clearance time: median slope half life for patients with recrudescence was 2.5 hours compared to 2.2 hours for patients without recrudescence. Similarly, mean(SD) were 2.8 (1.07) and 2.4(1.39) for each group (p=0.044). Of note, twenty four subjects had slope half life >5 hours (the clinical definition of resistance), with 4% (2 of 47) occurring in the subjects with recrudescence and 2% (22 of 1112) occurring in the subjects without recrudescence (p=0.283).

Similar to the results for the slope half life, the time to clear all parasites was slightly longer for the patients with recrudescence 27.5 hours and 24.0 for patients without recrudescence.

3.4. Correlates and Risk Factors for Recrudescence

A logistic regression analysis was conducted to investigate the influence of risk factors such as slope half life, baseline parasitemia, treatment group, sex and surrogates for host immunity (age, seasonality, estimated endemicity, geographic location and number of previous malaria episodes) on recrudescence. In the univariate analysis – slope half life, seasonality, treatment group, site, and estimated endemicity each revealed a significant influence or association with recrudescence; and age each had a marginal influence (Table 5.4). Of note, when modeling site, due to the many sites (n=11) and the smaller centers - many geographic areas were combined to allow for a meaningful analysis. Only 5 subjects were enrolled in Burkina Faso. These subjects were combined with subjects from the neighboring country of Ghana for analysis. To allow estimation for Kenya and Tanzania which had small centers - all centers within each country were combined for analysis.

In the multivariate analysis shown on Table 5.5, slope half life ($p<0.001$, OR 4.89; 95% CI 2.20-10.86), treatment group ($p=0.004$; OR 4.13; 95% CI 1.58, 10.77), seasonality ($p=0.008$; OR 3.76; 95% CI 1.41-10.02) and estimated endemicity ($p=0.018$; OR 2.27; 95% CI 1.15-4.49) remained important factors influencing recrudescence. Age ($p=0.076$) and sex ($p=0.106$) appear to have a marginal contribution. Site and Country did not provide any important additional information once estimated endemicity entered the model and were not selected in the final model.

Delayed parasite clearance time, malaria occurring during the rainy season, treating with CDA, and coming from an area of high estimated endemicity (high transmission) appear to be associated with higher recrudescence. Younger children (< 5 years) and females also appear to be more likely to experience recrudescence.

4. Discussion

The primary objective of this study was to describe the risk factors and in particular slope half life, treatment, and surrogates for host immunity and to assess if recrudescence may be explained by these factors. Delayed parasite clearance time, as assessed by longer slope half life, has been associated with development of resistance. By definition a slope half life > 5 hours indicates resistance. It is not clear however if delayed parasite clearance times (not meeting the definition of resistance) might also influence the later return of the initial parasite infection, e.g. recrudescence.

In order to explore whether parasite clearance time was associated with recrudescence, slope half life was included in this analysis. Slope half life which is the time in hours needed to clear 50% of the initial parasites is one of the common measures for assessing malaria treatment success. In the past however different definitions and individual interpretations as to whether individual patient profiles were a good model fit complicated the use of this measure when trying to compare data. With the availability of the standardized calculations from the WWARN Parasite Clearance Estimator (PCE) this will advance the use of this measure across the study of malaria. Since this is a recent assessment and has not been widely explored with regard to recrudescence at this time, including this in the current research may offer new insight. Using the PCE tool for this analysis offers advantages for this research allowing consistent definitions and computations which can be compared to other studies and data over time. This measure of slope half life also allows an assessment of clearance that is not dependent on the baseline parasitemia level – which offers an additional aspect not previously addressed.

Slope half life had a significant contribution for this analysis. The interpretation of this finding is also of interest since slope half life >5 hours has been used to assess the occurrence of resistance. Patients with slope half life >5 hours occurred in both the group with recrudescence

and the group without recrudescence. A slightly higher percentage occurred in the group with recrudescence. However, this was only 2 patients and therefore should be interpreted with caution.

Treatment group also had a significant contribution for this analysis. The artemisinin component of the ACT clears the parasites quickly for both treatment arms. However, the partner drug (CD vs. L) which is supposed to offer the longer term protection for the combination therapy due to the longer half-life of the partner drug appeared to show some difference. This does highlight the need to consider not all ACTs are equally effective and the choice of partner drug may have implications for the resistance profile as well as recrudescence [34, 52, 102, 117, 118].

Other results of this analysis appear to concur with previous literature from other regions - finding factors associated with host immunity such as age, seasonality, estimated endemicity to be important risk factors for recrudescence in this study population as well [3, 29, 74].

Previous studies have also indicated baseline parasitemia as a prominent risk factor for recrudescence (Ittarat, 2003; Dorsey, 2004). Although patients with very low parasitemia appeared to be less likely to have recrudescence, baseline parasitemia did not appear as a strong risk factor in this study.

Though the current study offers meaningful and relevant results assessing risk factors for recrudescence in patients treated with ACTs in SSA - some limitations are described below.

The study population is limited to the specific centers and countries studied, the specific treatments included, and the inclusion/exclusion criteria for this study. The results are limited to the data and covariates collected and analyzed in these studies. The inclusion of additional factors might provide different results in combination with the factors presented and possibly result in a different overall model.

Some of the derived data for this study such as seasonality and estimated endemicity may be considered ‘soft’ data and while it is helpful for exploratory research would require a more

directed and precise assessment for future investigative analyses. Estimated endemicity data was based on available maps from 2010, which may have differed from Endemicity data in 2007 had this been available. Also, the estimated endemicity data is based on modeling to estimate endemicity across regions and was not intended to provide point estimates for specific locations. Similarly, seasonality was based on typical months of the rainy and dry seasons for each country from travel data and maps available from Mapping Malaria Risk in Africa [48] and again is providing a general assessment and not intended to provide accurate rainfall amounts for precise locations. In addition, the start of a rainy season may not indicate the actual start of intense malaria transmission – which may be days or weeks into the rainy season.

Both of these variables were also based on geographic location or site to make these assessments and therefore a correlation with site is expected when analyzing this data.

Slope half life was calculated using the standardized PCE tool available from WWARN. For several cases the slope half life was not able to be calculated and remained as a missing data point. Since the multivariate modeling requires data present for all of the candidate risk factors, the subset of data used for the multivariate modeling may have differed from the full set of data, although initial comparisons of summary statistics for the two sets were similar.

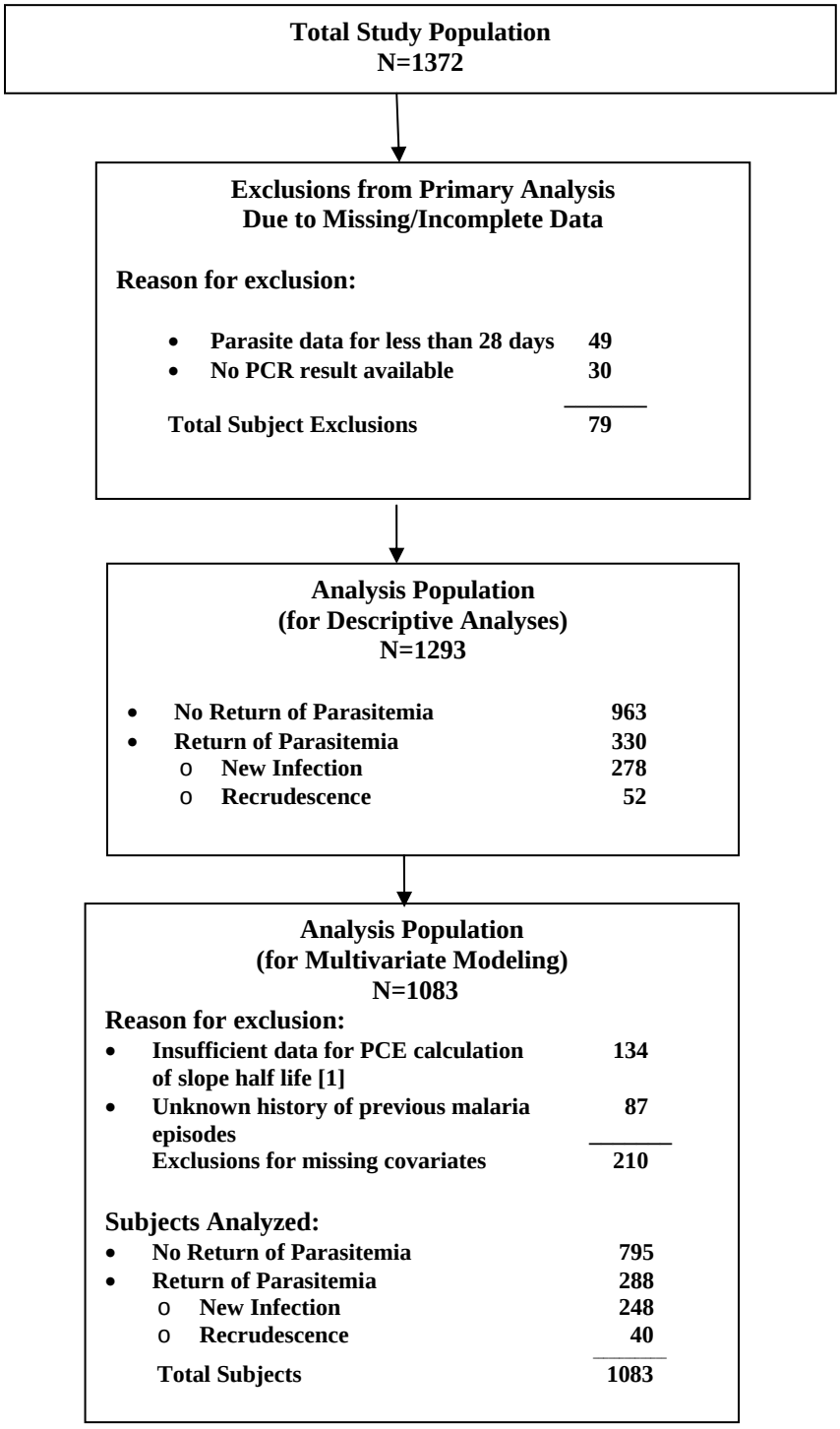
It is also important to mention the possibility for misclassification errors to have occurred when assessing the PCR results to determine if cases were recrudescent cases versus new infections. At the time the study was conducted, the use of MSP-1, MSP-2, and GLURP methodology was the gold standard for review. However, more sophisticated methods are now available and studies suggest the older methods did lead to misclassification errors – especially in high transmission areas [69]. The impact this may have had on the current analyses is unknown – although the results do appear to show separation and trends consistent with the expected classification of this data.

5. Conclusions

Slope half life and treatment along with factors related to host immunity, may be a plausible explanation for the occurrence of recrudescence in sub-Saharan Africa. For this analysis, slope half life, treatment group, and risk factors related to host immunity (seasonality and estimated endemicity) were associated with the occurrence of recrudescence. Delayed parasite clearance (assessed as slope half life) was associated with increased recrudescence. However, very few recrudescent cases had a slope half life greater than 5 hours (the clinical definition of resistance). This suggests that recrudescence is not inherently related to artemisinin resistant parasite strains.

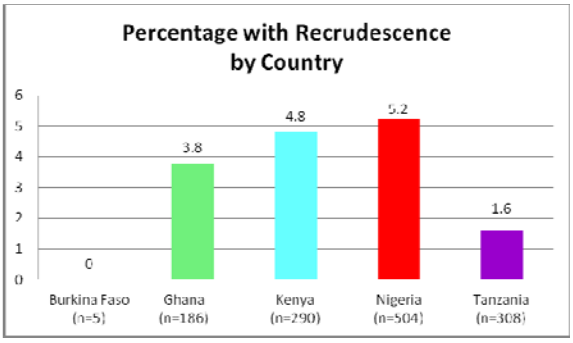
6. Tables and Figures

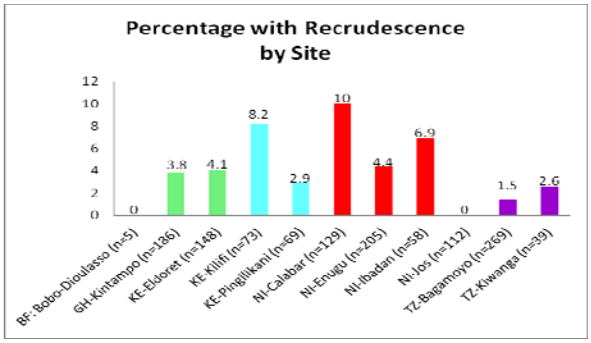
Figure 5.1. Consort Diagram

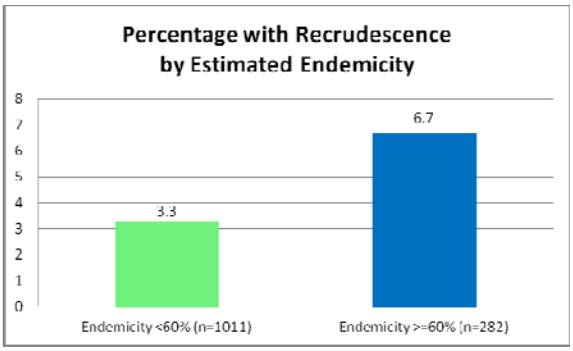


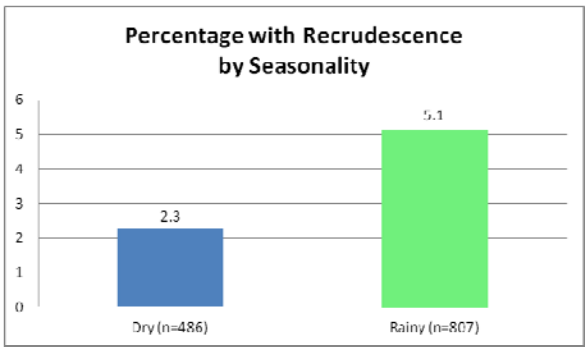
[1] Analyses using WWARN Parasite Clearance Estimator (PCE)

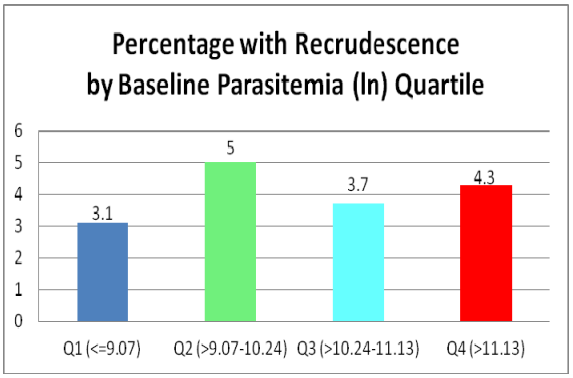
Figure 5.2. Percentage of Subjects with Recrudescence, by Risk Factor

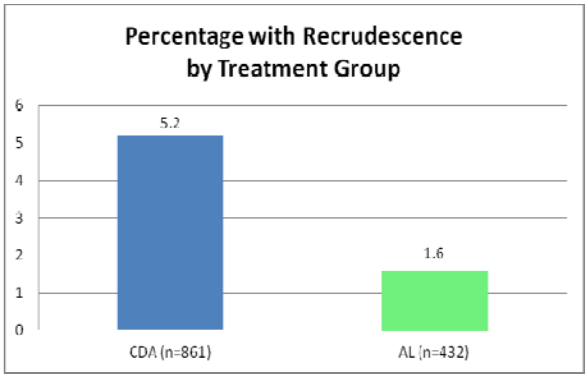


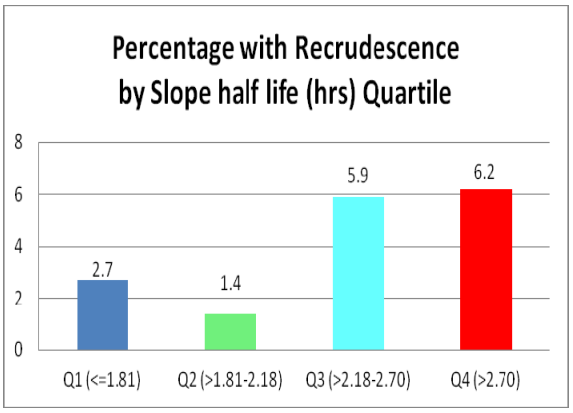












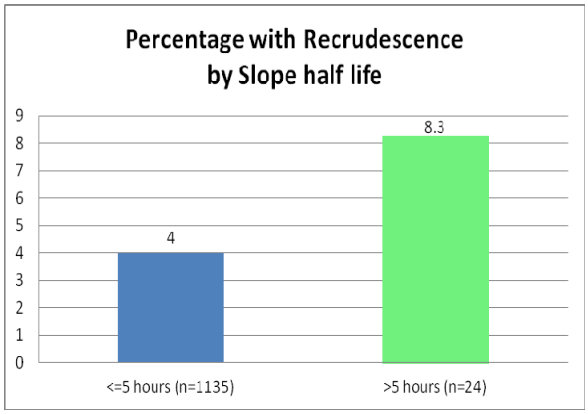


Table 5.1. Baseline Characteristics

	Total (n=1293)
Age (years)	
Mean (sd)	**4.2 (2.92)**
Median	**3.0**
Min – Max	**1-14**
Age Group	
<5 years	**835 (65%)**
5 to 15 years	**458 (35%)**
Sex	
Female	**634 (49%)**
Male	**659 (51%)**
Country	
Burkina Faso	**5 (<1%)**
Ghana	**186 (14%)**
Kenya	**290 (22%)**
Nigeria	**504 (39%)**
Tanzania	**308 (24%)**
Seasonality	
Dry	**486 (38%)**
Rainy	**807 (62%)**
Estimated Endemicity	
Mean (sd)	**0.5 (0.09)**
Median	**0.4**
Min – Max	**0.3 – 0.6**
Previous Malaria Episode	
Yes	**729 (56%)**
No	**477 (37%)**
Unknown	**87 (7%)**
Baseline Parasite Count	
Mean (sd)	**49,317 (58,188.1)**
Median	**27,967**
Min – Max	**0-705,600**
Baseline Parasite Count (ln)	**10.1 (1.42)**
Mean (sd)	**10.2**
Median	**0-13.5**
Min – Max	

Table 5.2. Classification and Timing of Recurrence (Including: Recrudescence and New Infections)

Classification of Patients with Recurrence

Classification	Number of Patients (%) n=330
Recrudescence	52 (16%)
Recrudescence only	31 (9%)
Recrudescence plus New Infection	21 (6%)
New Infection only	278 (84%)

Recurrence classification based on PCR Result assessed with *MSP-1, MSP-2, GLURP*

Recurrence and Overall Compliance

	Recrudescence only n=31	Recrudescence + New Infection n=21	New Infection only n=278
Compliant	27 (87%)	19 (90%)	268 (96%)
Noncompliant	4 (13%)	2 (10%)	10 (4%)

Patient received correct treatment and dose for all 3 dosing days.

Recurrence and Slope half life (hours)

Slope half life	Recrudescence only n=29	Recrudescence + New Infection n=18	New Infection only n=263
Mean (SD)	2.9 (1.15)	2.7 (0.95)	2.5 (1.30)
Median	2.5	2.4	2.2
Min-Max	1.4-5.9	1.7-4.8	1.3-18.2
Quartile 1 (<=1.81)	3 (10%)	5 (28%)	56 (21%)
Quartile 2 (>1.81 to <=2.18)	3 (10%)	1 (6%)	75 (29%)
Quartile 3 (>2.18 to <=2.70)	12 (41%)	5 (28%)	69 (26%)
Quartile 4 (>2.70)	11 (38%)	7 (39%)	63 (24%)
Slope half life <=5	27 (93%)	18 (100%)	257 (98%)
Slope half life >5	2 (7%)	0	6 (2%)

Computed by WWARN PCE tool. (Slope half life was unable to be calculated for 20 subjects.)

Timing of Recurrence

Scheduled Timepoint	Recrudescence only n=31	Recrudescence + New Infection n=21	New Infection only n=278
Day 7	2 (6%)	1 (5%)	2 (1%)
Day 14	3 (10%)	2 (10%)	7 (3%)
Day 28	17 (55%)	11 (52%)	151 (54%)
Day 42	9 (29%)	7 (33%)	117 (42%)
Unknown	0	0	1 (<1%)

Unscheduled assessments between visits are reported with the next Scheduled Day.

Table 5.3. Risk Factors for Recrudescence

	No Recrudescence (n=1241)	Recrudescence (n=52)	Row Percent[1]
Age (years)			
Mean (sd)	**4.2 (2.95)**	**3.4 (2.02)**	
Median	**3.0**	**3.0**	
Min – Max	**1-14**	**1-9**	
Age Group			
<5 years	**797 (64%)**	**38 (73%)**	**5%**
5 to 15 years	**444 (36%)**	**14 (27%)**	**3%**
Sex			
Female	**604 (49%)**	**30 (58%)**	**5%**
Male	**637 (51%)**	**22 (42%)**	**3%**
Country			
Burkina Faso	**5 (<1%)**	**0**	**0**
Ghana	**179 (14%)**	**7 (13%)**	**4%**
Kenya	**276 (22%)**	**14 (27%)**	**5%**
Nigeria	**478 (39%)**	**26 (50%)**	**5%**
Tanzania	**303 (24%)**	**5 (10%)**	**2%**
Site			
Burkina Faso: Bobo-Dioulasso	**5 (<1%)**	**0**	**0**
Ghana: Kintampo	**179 (14%)**	**7 (13%)**	**4%**
Kenya: Eldoret	**142 (11%)**	**6 (12%)**	**4%**
Kenya: Kilifi	**67 (5%)**	**6 (12%)**	**8%**
Kenya: Pingilikani	**67 (5%)**	**2 (4%)**	**3%**
Nigeria: Calabar	**116 (9%)**	**13 (25%)**	**10%**
Nigeria: Enugu	**196 (16%)**	**9 (17%)**	**4%**
Nigeria: Ibadan	**54 (4%)**	**4 (8%)**	**7%**
Nigeria: Jos	**112 (9%)**	**0**	**0**
Tanzania: Bagamoyo	**265 (21%)**	**4 (8%)**	**1%**
Tanzania: Kiwangwa	**38 (3%)**	**1 (2%)**	**3%**
Seasonality			
Dry	**477 (38%)**	**11 (21%)**	**2%**
Rainy	**766 (62%)**	**41 (79%)**	**5%**
Estimated Endemicity (%)			
<60%	**978 (79%)**	**33 (63%)**	**3%**
>=60%	**263 (21%)**	**19 (37%)**	**7%**

[1] Row percent is the percent of subjects with recrudescence for each sub-category.

Table 5.3. Risk Factors for Recrudescence (continued)

	No Recrudescence (n=1241)	Recrudescence (n=52)	Row Percent[1]
Previous Malaria Episode			
Yes	**698 (60%)**	**31 (69%)**	**4%**
No	**463 (40%)**	**14 (31%)**	**3%**
Treatment Group			
CDA	**816 (66%)**	**45 (87%)**	**5%**
AL	**425 (34%)**	**7 (13%)**	**2%**
Baseline Parasite Count (ln)			
Mean (sd)	**10.1 (1.43)**	**10.2 (1.29)**	
Median	**10.2**	**10.2**	
Min-Max	**0-13.5**	**7.0-12.2**	
Baseline Parasite Count (ln) Quartiles			
Quartile 1 (<= 9.07)	**314 (25%)**	**10 (19%)**	**3%**
Quartile 2 (> 9.07 – 10.24)	**307 (25%)**	**16 (31%)**	**5%**
Quartile 3 (> 10.24 – 11.13)	**311 (25%)**	**12 (23%)**	**4%**
Quartile 4 (> 11.13)	**309 (25%)**	**14 (27%)**	**4%**
Baseline Parasite Count (ln)			
<= Median (<=10.24)	**621 (50%)**	**26 (50%)**	**4%**
>Median (>10.24)	**620 (50%)**	**26 (50%)**	**4%**
Slope half life (hours)	**(n=1112)**	**(n=47)**	
Mean (sd)	**2.4 (1.39)**	**2.8 (1.07)**	
Median	**2.2**	**2.5**	
Min-Max	**1.2-28.0**	**1.4-5.9**	
Slope half life (hours) Quartiles			
Quartile 1 (<= 1.81)	**286 (26%)**	**8 (17%)**	**3%**
Quartile 2 (>1.81 to <=2.18)	**283 (25%)**	**4 (9%)**	**1%**
Quartile 3 (>2.18 to <=2.70)	**270 (24%)**	**17 (36%)**	**6%**
Quartile 4 (>2.70)	**273 (25%)**	**18 (38%)**	**6%**
Slope half life (hours)			
<= Median (2.18)	**569 (51%)**	**12 (26%)**	**2%**
>Median (2.18)	**543 (49%)**	**35 (74%)**	**6%**
Slope half life (hours)			
<=5 hours	**1090 (98%)**	**45 (96%)**	**4%**
>5 hours	**22 (2%)**	**2 (4%)**	**8%**

[1] Row percent is the percent of subjects with recrudescence for each sub-category.

Table 5.4. Logistic Regression Analysis - Univariate Analysis

Parameter(s)	Odds Ratio (95% CI)	p-value
Age (<5 vs. 5-15 years)	**1.51 (0.81, 2.82)**	**0.194**
Sex (Female vs. Male)	**1.44 (0.82, 2.52)**	**0.205**
Estimated Endemicity (>=60% vs. <60%)	**2.14 (1.20, 3.83)**	**0.010**
Seasonality (Rainy vs. Dry)	**2.31 (1.18, 4.54)**	**0.015**
Previous Malaria Episodes (Yes vs. No)	**1.47 (0.77, 2.79)**	**0.241**
Country		**0.102**
Country(Burkina Faso/Ghana vs. Tanzania)	**2.31 (0.72, 7.37)**	
Country(Kenya vs. Tanzania)	**3.07 (1.09, 8.65)**	
Country(Nigeria vs. Tanzania)	**3.30 (1.25, 8.68)**	
Site		**0.006**
Site (Burkina Faso/Ghana vs. Tanzania)	**2.31 (0.72, 7.37)**	
Site (Kenya vs. Tanzania)	**3.07 (1.09, 8.65)**	
Site (Nigeria: Calabar vs. Tanzania)	**6.79 (2.37, 19.47)**	
Site (Nigeria: Enugu vs. Tanzania)	**2.78 (0.92, 8.43)**	
Site (Nigeria: Ibadan/Jos vs. Tanzania)	**1.46 (0.39, 5.51)**	
Baseline Parasite Count(ln) (>10.24 vs. <=10.24, median value)	**1.00 (0.58, 1.75)**	**0.996**
Slope half life (hours) (>2.18 vs. <=2.18, median value)	**3.06 (1.57, 5.95)**	**0.001**
Treatment Group (CDA vs. AL)	**3.35 (1.50, 7.49)**	**0.003**

Outcome: Recrudescence (Yes vs. No)

Table 5.5. Logistic Regression Analysis - Multivariate Analysis

Parameter(s)	Odds Ratio (95% CI)	p-value
Slope half life (>2.18 vs. <=2.18 hrs)	**4.89 (2.20, 10.86)**	**<0.001**
Treatment Group (CDA vs. AL)	**4.13 (1.58, 10.77)**	**0.004**
Seasonality (Rainy vs. Dry)	**3.76 (1.41, 10.02)**	**0.008**
Estimated Endemicity (>=60% vs. <60%)	**2.27 (1.15, 4.49)**	**0.018**
Age Group (<5 vs. 5-<15 years)	**1.98 (0.93, 4.22)**	**0.076**
Sex (Female vs. Male)	**1.73 (0.89, 3.35)**	**0.106**

Outcome: Recrudescence (Yes vs. No)

CHAPTER 6: SUMMARY

1. Summary of Findings

The specific aims of this research were to explore potential determinants of parasite clearance time and recrudescence and to assess if the determinants would be indicative of host-related measures such as immunity or parasite-related such as resistance.

Study Aim 1 – Determinants of Parasite Clearance Time

The results from this analysis indicated site ($p<0.001$) and baseline parasitemia ($p<0.001$) were significant factors in explaining the differences in parasite clearance time (slope half-life <= 5 hours vs. > 5 hours). 24 subjects met the definition of artemisinin resistance (slope half-life > 5 hours) with approximately half of these subjects (13 of 24) coming from one particular site in Nigeria.

The data collected for this study prior to the widespread use of ACTs in this region offered a unique opportunity to uncover the occurrence of delayed PCT which would not be tied to therapy-induced resistance, but may be part of a natural selection process inherent in geographic regions.

Some delayed PCT pre-existed the introduction of artemisinin combinations in Africa. This suggests that resistant parasite strains, particularly in some parts of Africa, pre-existed the introduction of ACTs.

Study Aim 2 – Determinants of Recrudescence

The results from this analysis indicated slope half life ($p<0.001$, OR 4.89; 95% CI 2.20-10.86), seasonality ($p=0.008$; OR 3.76; 95% CI 1.41-10.02), treatment group ($p=0.004$; OR 4.13; 95% CI 1.58, 10.77) and estimated endemicity ($p=0.018$; OR 2.27; 95% CI 1.15-4.49) were

important factors influencing recrudescence. Age (p=0.076) and sex (p=0.106) appeared to have a marginal contribution.

Slope half life and treatment along with factors related to host immunity, may be a plausible explanation for the occurrence of recrudescence in sub-Saharan Africa. For this analysis, longer slope half life (time needed to clear 50% of parasites), treatment group (CDA), and risk factors related to host immunity (seasonality – occurrence during the rainy season; and estimated endemicity – high endemicity) were associated with the occurrence of recrudescence. Delayed parasite clearance (assessed as slope half life > 2.18 hours, the median value) was associated with increased recrudescence. However, very few recrudescent cases (2 of 52) had a slope half life greater than 5 hours (the clinical definition of resistance). This suggests that recrudescence is not inherently related to artemisinin resistant parasite strains.

2. Strengths and Limitations

The current research offers many strengths to assess the determinants of delayed parasite clearance time and recrudescence in patients treated for uncomplicated *P. falciparum* malaria in SSA - including the use of data from a large well-controlled clinical trial conducted to Good Clinical Practice (GCP) standards. Collecting blood samples in-clinic at standard times, every 8 hours provided meaningful data to obtain accurate assessments of the parasite clearance time. The controlled in-clinic environment offered a controlled setting to avoid or reduce the contamination of new infections (new mosquito bites) occurring during the initial period. The large study size and multiple regions also offered the ability to explore several risk factors for the analyses.

The research and analyses also utilized the standardized Parasite Clearance Estimator (PCE) from the WWARN website, so the results will offer a consistent measure which can be compared to other studies with similar measures and ensure a consistent application of the definition of resistance measured as slope half-life > 5 hours.

Though, the current research offers meaningful and relevant results, some limitations should also be noted.

The study population is limited to the specific centers and countries studied, and the results are limited to data/covariates collected and analyzed in this study. The inclusion of additional factors might provide different results in combination with the factors presented and a different overall model.

The large dataset offered the opportunity to explore several risk factors for parasite clearance time and recrudescence.

Unfortunately the study did not collect samples which could be used to assess genetic markers of resistance which have recently been identified. Similarly, hemoglobin type which has been associated with delayed PCT also was not collected in this study and could not be assessed.

As noted previously, the derived data for this study such as seasonality and estimated endemicity may be considered 'soft' data and while it is helpful for exploratory research would require a more directed and precise assessment for future investigative analyses.

Only two particular ACTs are included in the study so caution may be needed if generalizing the results to all ACTs. Also, the study was conducted in 2007 and reported in 2009 which is prior to the widespread use of ACTs in SSA. In this case artemisinin resistance introduced by the ACT may not have had a chance to develop at this time.

Though missing data was kept to a minimum for this study due to good study conduct and quality control measures, the following limitations should be noted with regard to the outcome assessments. For assessing the slope half-life, approximately 10% of the data was missing due to the inability to get an appropriate fit of the data for the PCE model. When assessing recrudescence vs. new infection, as noted previously, it is possible that misclassification errors could have occurred if a new infection was genetically similar to the original infection. This would result in a

'new infection' being misclassified as a 'recrudescence' or failure and could therefore dilute the results or differences between the groups.

It also should be noted that the parent study from which the data for this ancillary study is derived, enrolled only subjects without known G6PD deficiency (based on inclusion criteria for the parent protocol). Though this selection was relevant since the planned treatment was contra-indicated in patients with 'known' G6PD deficiency and represents a 'real-world' treatment setting, there are limitations to the inference for G6PD deficient subjects in general as the subjects were a pre-selected subset for this study.

Similarly, the study inclusion/exclusion criteria did not allow patients with other underlying disease; co-infections; malnutrition, or severe/complicated malaria to be entered in the study. Therefore the generalizability of the study is limited to the characteristics of the inclusion criteria.

3. Public Health Implications

This research identified several patients meeting the definition of artemisinin resistance and possibly exhibiting pre-existing or naturally occurring resistant parasite strains in sub-Saharan Africa. This is an important finding which may impact surveillance for resistance in SSA.

The finding indicating slope half life is associated with recrudescence along with other host factors is useful information to show recrudescence is not only indicative of resistance and may be helpful in treating patients.

4. Future Research Directions

The planned analyses were relevant and informative for assessing the determinants of parasite clearance time and recrudescence. A recommendation for future research would be to extend the study to also include genetic markers.

The suggestion that resistant parasite strains, particularly in some parts of Africa, pre-existed the introduction of ACTs is an important finding which may have implications for treatment options in the future. The implications for the existence of naturally occurring variation which may impact treatment effectiveness will be important to explore and consider for future research. These 'pockets' or areas of naturally occurring variation have been cited [3, 116]. Studying the parasites in these unique geographic areas could lead to a better understanding of the origins of resistance and to better surveillance tools to prevent its spread.

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