Challenges to the Treatment of Malaria

Kimberly A. Porter

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Approved by:

Steven Meshnick, Chair

Christina Burch

Stephen Cole

Joseph Eron Jr.

Jonathan Juliano

Charles Poole III

ABSTRACT

KIMBERLY A. PORTER: Challenges to the Treatment of Malaria
(Under the direction of Steven Meshnick)

Malaria remains a significant cause of morbidity and mortality. Successful treatment of malaria is threatened by widespread drug resistance and co-infections with HIV.

This dissertation explored two challenges to malaria treatment. The first aim addressed outcome misclassification in antimalarial treatment trials. Without accurate classification of patients' outcomes, estimates of drug efficacy are flawed. We identified factors related to outcome misclassification: transmission intensity, the distribution of genetic variants in parasite populations, multiplicity of infection, and PCR-insensitivity to minority variants; then used our findings to develop a Monte Carlo uncertainty analysis.

Using the uncertainty analysis, we found that misclassification of new infections as treatment failures was common and underestimated treatment efficacy in the high transmission area. The initial estimate of the cure rate in the high transmission area was 63.8%; after adjustment for uncertainty related to outcome misclassification, the 95% simulation interval of the cure rate was 74.6 to 83.3%. The initial estimate of the cure rate in the low transmission area was 94.0%; after the

uncertainty adjustment the 95% simulation interval of the cure rate was 93.5 to 96.5%.

The second aim was to assess the effect of a co-formulation of HIV protease inhibitors (PI) on incidence of clinical malaria among HIV-infected adults. Laboratory evidence has demonstrated that HIV PIs inhibit growth of *Plasmodium falciparum*, a malaria-causing parasite. We conducted an ancillary analysis of data collected by the Adult AIDS Clinical Trials Group in two trials comparing PI-based against non-nucleoside reverse transcriptase inhibitor (NNRTI)-based antiretroviral therapy on the incidence of clinical malaria in study participants residing in areas with endemic malaria.

We used pooled logistic regression to calculate hazard ratios (HR) and 95% confidence intervals (CI). There was no effect of PI-based therapy on incidence of clinical malaria (HR = 1.03, 95% CI (0.74 - 1.44)), nor was there modification of the HR by seasonality and use of concomitant medications.

Successful treatment of malaria is a global health priority. This dissertation provides a novel way to estimate treatment efficacy and proposes that HIV PIs may not have antimalarial action in HIV-infected patients at risk of co-infection.

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For: *Factors influencing PCR-corrected cure rates in antimalarial efficacy trials*

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For: *The effect of HIV-1 protease inhibitors on incidence of malaria*

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EIR: entomological inoculation rate

MOI: multiplicity of infection

ACT: Artemisinin-based combination therapy

HIV: human immunodeficiency virus

PI: protease inhibitor

LRV: lopinavir

RTV: ritonavir

WHO: World Health Organization

PCR: polymerase chain reaction

***msp1*: merozoite surface protein 1**

***msp2*: merozoite surface protein 2**

***glurp*: glutamate rich protein**

MMV : Medicines for Malaria Venture

HTA : heteroduplex tracking assay

LPV/r: co-formulation of lopinavir and ritonavir

NNRTI: non-nucleoside reverse transcriptase inhibitor

AACTG: Adult AIDS Clinical Trials Group

RCT: randomized controlled trials

NVP: nevirapine

HR: hazard ratio

RNA: ribonucleic acid

ART: antiretroviral therapy

NRTI: nucleoside reverse transcriptase inhibitor

EFV: efavirenz

DTSA: discrete-time survival analysis

CI: confidence interval

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Malaria, a major cause of morbidity and mortality, is a mosquito-borne disease caused by *Plasmodium* parasites. In 2008, there were an estimated 243 million cases of malaria resulting in 863,000 deaths worldwide.(1) Malaria can be controlled through environmental modification and prevention strategies; it has been successfully eliminated from several regions of the world including the United States. In parts of the developing world, however, particularly Sub-Saharan Africa and Southeast Asia, the burden of malaria remains immense.

P. falciparum is the most pathogenic of the five human disease-causing *Plasmodium* species and is the focus of this work; from this point forward, malaria will refer exclusively to infection with *P. falciparum*. *P. falciparum*, which is transmitted by the *Anopheles* mosquito, is highly prevalent in Sub-Saharan Africa and is the most common *Plasmodium* species in tropical and subtropical regions.(2)

The life-cycle of *Plasmodium* is complex and requires both a mosquito and vertebrate host. Different *Plasmodium* species have slightly different life-cycles; the following refers to *P. falciparum*. In the mosquito, gametocyte-stage parasites ingested from the vertebrate host undergo sexual reproduction developing first into zygotes, then ookinetes, which eventually rupture releasing sporozoites. This is known as the

sporogonic cycle and lasts roughly two to three weeks. The sporozoites infect the vertebrate host through the salivary gland of the mosquito when it takes a blood meal. Once inside the vertebrate host, sporozoites infect liver cells where they develop into tissue schizonts. Each tissue schizont undergoes asexual replication generating merozoites; this is known the exo-erythrocytic cycle. Finally, the merozoite-stage parasites rupture the liver cell and infect red blood cells. There they undergo asexual reproduction and develop into immature trophozoites (referred to as ring-stage parasites). At that time, the parasites develop into either gametocytes or mature trophozoites which then develop into erythrocytic schizonts. The gametocytes are taken up by a feeding mosquito; the erythrocytic schizonts rupture and release merozoites which start the erythrocytic cycle again. (Figure 1)

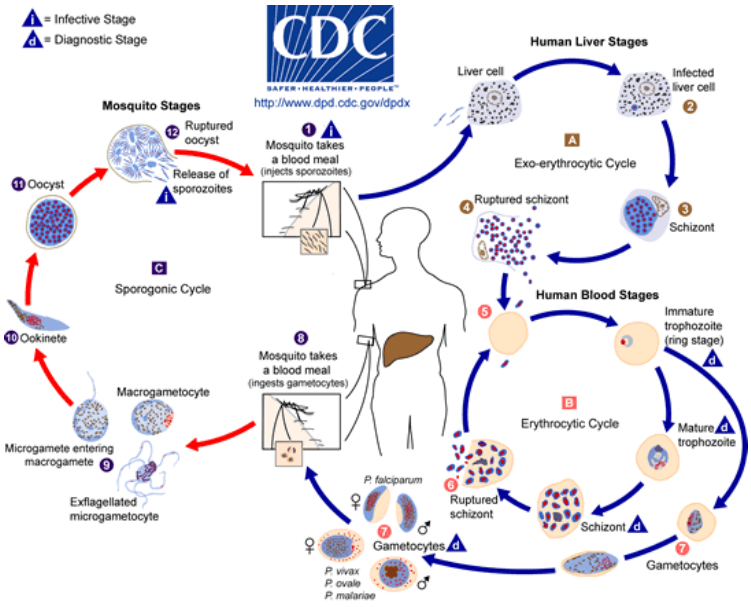

Figure 1. The life-cycle of *Plasmodium*.(3)

Malaria transmission intensity varies regionally and often seasonally. Transmission intensity is commonly measured by the entomological inoculation rate (EIR) and multiplicity of infection (MOI). The EIR estimates the number of

infectious bites per person-year and can differ greatly among locations. During the rainy season in Uganda's Tororo District, the EIR is estimated to be 591(4); in a semi-urban area in Burkina Faso, the EIR is believed to be under five.(5) MOI is the patient's number of infections (identified by the number of genotypes in a blood sample) and is positively correlated with transmission intensity. It is also inversely associated with the host's level of acquired immunity.(6) Acquired immunity provides protection against malaria in individuals with regular exposure, i.e. those living in areas of stable or high transmission. In such areas, clinical disease is far more common in children who do yet possess sufficient immunity, and pregnant women, as pregnancy reduces immunity to malaria.(7) In low transmission areas, less immunity develops and clinical disease is more likely to occur at any age.(8)

Host defenses associated with acquired immunity help protect against infection, control levels of parasitemia and reduce incidence of clinical disease.(9) It is believed that this is in large part due to humoral responses with antibodies appearing to target different parasite stages.(2)

In addition to the specificity of antibodies to different parasite stages, evidence suggests that acquired immunity may be strain-specific.(10) Acquired immunity takes years to develop and is dependent upon transmission intensity; it does not alleviate the need for effective antimalarial treatments.

Antimalarial drugs act against different parasite stages. Many antimalarials target blood stage parasites. These drugs include quinine, chloroquine, and the artemisinin derivatives (Arteether, Artesunate, Artemether). Others target other parasite stages;

primaquine has anti-gametocyte action. Some drugs appear to target more than one stage. For example, primaquine also acts against tissue schizonts.(2)

Antimalarials also have different half-lives and each may be particularly useful in certain circumstances. Treatments with long half-lives, such as mefloquine, may confer longer lasting protection and be especially important in areas of high transmission as they may be better able to decrease reinfection.(11) Drugs that are highly efficacious against primary blood stage infection or have shorter half-lives, such as chlorproguanil/dapsone (LapDap) and the artemisinin derivatives, may play a particularly important role in reducing the clinical illness that occurs during primary infections.(11)

Current malaria treatment strategies employ combinations of drugs to help slow development of drug resistance. Artemisinin-based combination therapy (ACT) has demonstrated high efficacy; it consists of a short-lived, fast-acting artemisinin derivative partnered with a drug with a longer half-life to kill remaining parasites.(12) Care must be taken to select partner drugs to which parasites in the region remain sensitive.

Determining which treatments remain effective can be done through clinical trials, public health surveillance and observational epidemiologic studies. Much of this work is carried out in areas with high EIR; therefore to accurately assess the drug's effect, these approaches require the ability to distinguish between reinfection and recrudescence. Resistance can also be detected through *in vitro* testing.(13)

Antimalarial resistance threatens the success of malaria treatment programs. Effective treatment of malaria is also complicated by the biological, geographic, and therapeutic interactions between malaria and Human Immunodeficiency Virus (HIV) which will discussed in greater detail in following sections.

This dissertation examined factors relevant to malaria treatment in two distinct frameworks. The first aim was to explore the impact of misclassification on cure rates in antimalarial efficacy studies using simulations and to develop a Monte Carlo uncertainty analysis. The second aim was to quantify the effect of a co-formulation of two HIV-1 protease inhibitors (PIs), lopinavir (LPV) and ritonavir (RTV), on malaria incidence using a discrete-time survival analysis.

A. Factors influencing PCR-corrected cure rates in antimalarial efficacy trials

The World Health Organization (WHO) recommends that first-line antimalarial treatment policies be changed when a drug's cure rate falls below 90%.(14) The cure rate is the proportion of patients who recover and become aparasitemic after receiving treatment. To estimate that proportion, differentiating between reinfection and recrudescent infection is essential. Polymerase chain reaction (PCR)-correction of cure rates, that is, genotyping paired samples from patients before and after treatment to classify whether recurrent parasitemia is a new infection or reflects treatment failure, has been in use for more than 20 years. However, PCR-correction may produce erroneous results dependent upon the diversity of genetic markers in the local parasite population, the allelic frequency of those markers, and transmission intensity. The insensitivity of PCR to minority variants may also lead to misclassification.

1. Classification of recurrent parasitemia

a. PCR-correction

PCR involves the use of primers (sequences of DNA that are complementary to regions of the genetic marker of interest), nucleotides and a DNA polymerase to amplify segments of DNA to observable quantities; it allows researchers to determine which genetic variants are present in a biological sample. PCR-correction, the use of genotyping to distinguish between reinfection and recrudescent infection, is used to adjust cure rates ("cure rate" is commonly used in the literature and is the language we use here for consistency, however it is actually the proportion of patients who are treated successfully) in antimalarial efficacy studies. By comparing parasite variants present in the patient before and after treatment, researchers can decide if the patient cleared the initial infection and has become reinfected, or if she has not cleared her initial infection (recrudescence).

In a recent review, it was reported that the first use of PCR-correction was in 1997 and it has become increasingly common.(15) One of the earlier assessments of PCR-correction declared that to be successful in differentiating between reinfection and recrudescence, "the theoretical requirements would be: (1) ensured protection from additional mosquito bites; (2) coadministration of drugs effective against liver stages, such as primaquine; and (3) analysis of a sufficient number of consecutive samples."(16) In the absence of such an ideal setting, the authors suggested that adequately reliable results can be achieved with sufficient sampling, PCR efficiency, and sufficient resolution to identify different alleles.(16)

The importance of PCR-correction is well-documented and understood. In a review of antimalarial studies conducted from 1995 to 2005, 175 treatment arms were identified in which PCR-correction had been used. In 41 treatment arms (26%), new

infections were responsible for 50-74% of recurrent infections. In 36 treatment arms (23%), new infections were responsible for at least 75% of recurrent infections.(15) In another evaluation of data from multiple studies, the authors concluded, "Without PCR genotyping, 36% of the recurrent parasitemias after day 14 (260/696 recurrences) would have been wrongly classified as failures. This would have lead to 1,048 cases being considered failures by day 28 (352 by or before day 14 plus 696 between days 14 and 28), thus overestimating the risk of failure by about one-third."(17) Differences between crude and PCR-corrected failure rates greater than 10% have been reported elsewhere.(18) Not all studies have found that many episodes of recurrent parasitemia were the result of reinfection; one study found that all recurrent parasitemias were the result of recrudescence though in that study all 12 participants carried isolates resistant to both treatments. (19)

PCR-correction is not without limitation and misclassification of both reinfections and recrudescent infections can occur. It is possible that a new infection will be of the same variant as the first and the recurrent parasitemia will erroneously be classified as a recrudescent infection. Conversely, PCR may fail to identify all the genotypes in the pre-treatment sample and the recurrent parasitemia may be falsely classified as a new infection. Factors that play a role in these types of misclassification will be discussed in greater detail below.

b. Definitions of recrudescence

Different definitions of recrudescence appear in the literature; this changes analytical approaches with regard to how and which patients are considered when calculating the cure rate or other endpoint. The WHO recommends that markers sharing

even a single band indicate recrudescence but if any of the markers genotyped do not share a band, indicating a new infection, then that is the patient's classification.(20) Other definitions attempt a more nuanced approach. Kwiek *et al*. considered recurrent parasitemias that shared a single, highly prevalent band (appeared in more than 10% of samples) indeterminate, not recrudescent.(21) For indeterminate parasitemias, they used the frequency of the shared allele and the number of variants in recurrent samples to calculate an estimated probability of a chance-match.(21) Cattamanchi *et al*., who had genotyped *msp1* (the gene encoding merozoite surface protein 1) , *msp2* (the gene encoding merozoite surface protein 2), and *glurp* (the gene encoding glutamate rich protein), explored different ways of addressing "indeterminate" recurrences, those in which the patient had both shared and new alleles.(22) They considered three schemes: (1) all recurrences classified as recrudescent, (2) recurrent infections classified as reinfections if at least 50% of the post-treatment bands were new, or (3) all recurrences classified as reinfections. They found that using the second scheme generated hazard ratios most similar to their reference group (patients who had only shared or only new bands) and concluded, "Our analysis showed that the episodes initially classified as indeterminate were much more likely to be caused by reinfection than by recrudescence."(22)

Additionally, which samples are even subject to genotyping varies. In a review by Collins *et al*. they found that, "Most trials only genotyped samples from episodes of recurrent parasitemia that occurred after a defined number of days following therapy."(15) It is not uncommon for patients who fail within the first seven or even 14 days after treatment to automatically be considered recrudescent.(17, 18, 22) This may be

unwise as unexpectedly high numbers of new infections among recurrent parasitemias have been identified as early as Day 7 (19%) and Day 14 (47.1%).(23) Of course, these high numbers may also be the result of PCR-related misclassification of recrudescent infections.

c. WHO and Medicines for Malaria Venture (MMV) recommendations

The WHO recommends that antimalarial efficacy trials follow patients for no fewer than 28 days and that PCR-correction be used to differentiate reinfection from recrudescence.(20) In 2007, recommendations for standardizing PCR-correction were issued by a collaboration of the WHO and MMV(20); they included:

1. Definition of a **new infection**: Recurrent parasitemia "in which all alleles in the post-treatment sample…are completely different from those in the admission sample," for at least one loci (Figure 2).
2. Definition of a **recrudescent infection**: At each locus, one or more alleles are shared in pre- and post-treatment samples (Figure 2).

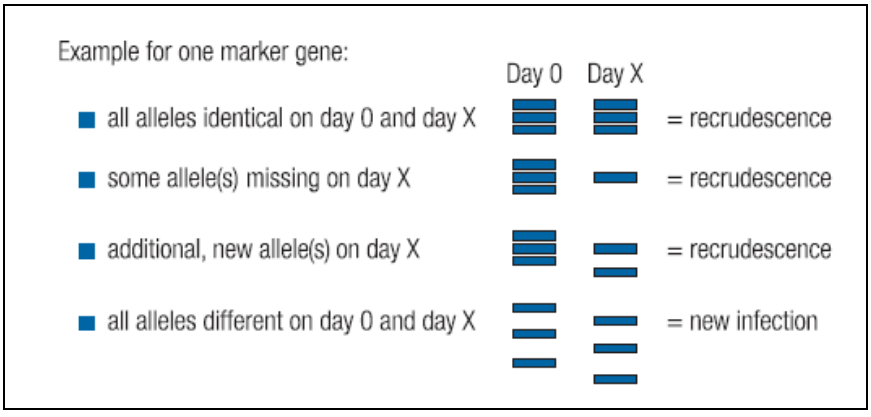

Figure 2. WHO/MMV definitions of recrudescence and reinfection. Used with permission.(20)

3. Only two samples are required (no consecutive sampling), one pre-treatment and one at the time of recurrent parasitemia.
4. Use of commercial blood collection cards as opposed to untreated filter paper (this helps to ensure the success of genotyping).
5. Use of capillary electrophoresis when possible (this increases the ability to distinguish between different alleles).
6. Stepwise (stopping when a new infection is detected) nested PCR genotyping of all recurrent parasitemias for *msp1*, *msp2*, and *glurp* (Figure 3).
7. Accreditation of laboratories to provide quality assurance.
8. When the PCR-corrected cure rate falls below 90%, they recommend collecting and reporting additional information: existence of gametocytes at the time of recurrent parasitemia, the average multiplicity of infection and the distribution of alleles in the parasite population. They state that these values can then be used to calculate chance-matches but do not make a specific recommendation for how to do this.

These recommendations are useful with regard to standardization, an important next step when considering the wide variety of genotyping techniques and definitions of recurrence, a shortcoming highlighted by Collins *et al*.(15) However, the recommendations do not address all of the technical and biological factors that affect PCR-correction. These factors are discussed below.

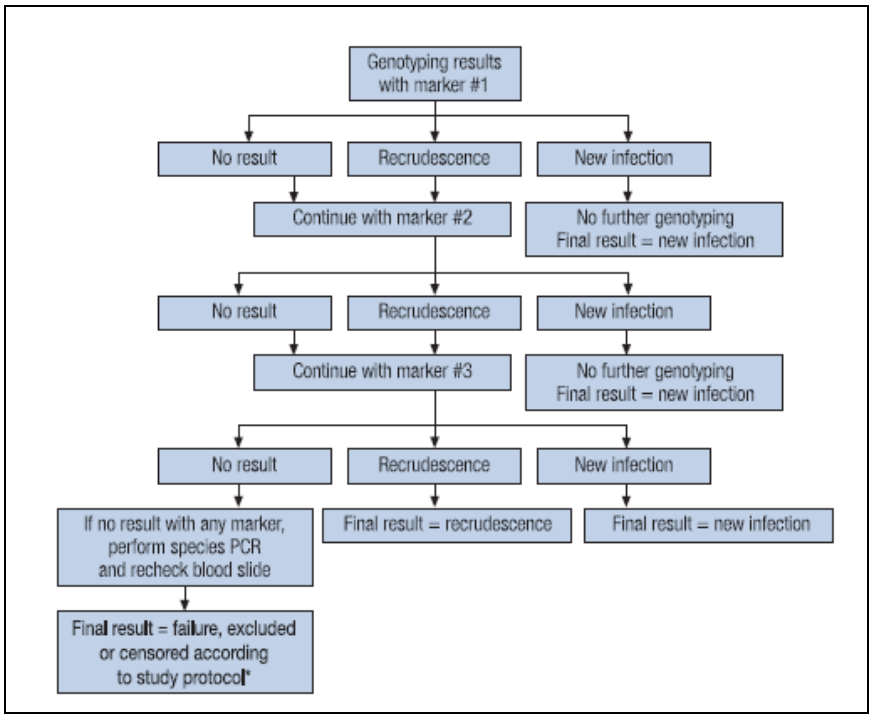

Figure 3. Stepwise genotyping with three markers. Used with permission. (20)

2. Technical considerations of PCR-correction

a. Selection of genetic markers

Frequently used markers for PCR-correction include *msp1*, *msp2* and *glurp*. They are considered useful because they: (A) contain variable regions that result in different sizes of PCR products, (B) tend to have high diversity within parasite populations, (C) and are single-copy genes on different chromosomes.

Microsatellites, non-coding repeated sequences of nucleotides, have been suggested as alternatives to traditional markers. The argument for their use includes the possibility that msp-coding genes and *glurp* may be under immune selective pressure.(10) In a study of microsatellite use, researchers compared analysis of a single polymorphic microsatellite to analyses of *msp1* and *msp2*. They found that the detection threshold was

similar to that of *msp1* and an order of magnitude lower than *msp2*. From a total of 69 samples, 46 (67%) had been classified as recrudescent by using a combination *msp1* and *msp2*; when the samples were then evaluated for the microsatellite, 23 of those 46 samples (50%) appeared to be new infections. Seven of 30 samples (23%) identified as recrudescent by microsatellite analysis were new infections according to analysis by the msp genes.(10)

In addition to which markers are used, the number of markers must be decided upon. Though an analysis of multiple studies found that, "use of at least three genotyping markers was not found to increase the odds of classification as new infection...", (15) many have argued for the use of multiple markers. This is primarily due to the increased allelic diversity afforded by multiple markers which may be especially important in low transmission areas where parasite diversity is thought to be low.(22) The benefits of multiple markers need to be balanced against cost and the likelihood of results that are difficult to interpret.(22) Additionally, the use of multiple markers increases the probability that at one locus PCR will fail to detect all pre-treatment genotypes possibly leading to misclassification of a true recrudescent infection.(5) A stepwise approach to genotyping in which no additional markers are evaluated after one identifies a new infection has been used and is recommended by the WHO and MMV.(20)

b. Consecutive-day sampling

Using a single pre-treatment and single post-treatment sample may result in an incomplete description of a patient's infection resulting in outcome misclassification if within-host parasite population dynamics are highly changeable as they are in

asymptomatic patients.(24) Whether population dynamics are similar in symptomatic patients has been the subject of some debate.

In a study of 13 cases of malaria who returned to Sweden after travel in malaria-endemic areas and had blood samples taken at 12 hour intervals for a minimum of three days during and after treatment, 12 (92%) had the same genotypes in all samples (in some patients, the post-treatment samples had only a subset of the pre-treatment genotypes).(25) This indicated that multiple samples may be less necessary in symptomatic patients when determining their parasite populations. However, the authors did allow that, "follow-up analysis in drug trials distinguishing recrudescent parasites from new infections may still be favored by analysis of additional samples…since an asymptomatic parasitemia may confer dynamics other than the infection in the acute phase and a single sample may then only partly reflect the infection parasite population."(25) In another study in which samples were drawn on Day 0 – 3, 7, 14, 21, 28, 35, 42 and any day of recurring illness, standard single sample analysis performed similarly well to repeated sampling, identifying 27 of 33 recrudescences (82%) when genotyping *msp2* and 17 of 21 (81%) when genotyping both *msp1* and *msp2*.(26) However these authors also chose to recommend more than one pre- and post-treatment sample, suggesting instead that samples be taken on two consecutive days at the beginning of follow-up and on two consecutive days at the time of recurrent parasitemia.(26) Stronger support for the use of multiple samples came from a study that used a quantitative fragment-analysis, as opposed to standard PCR methods. They found that parasite populations within symptomatic patients were highly changeable; 14 of 20

patients (70%) had clones that "showed major fluctuations," with some clones disappearing and reappearing within two hours.(27)

c. Sensitivity and resolution

Differentiating between two alleles is a requirement of distinguishing between reinfection and recrudescence. The WHO/MMV genotyping recommendations provide guidance on the extraction of DNA and storage of samples (20); these issues certainly play an important role in the efficiency and sensitivity of PCR-correction. For the purposes of this project, other factors affecting the identification of distinct alleles, namely PCR sensitivity and resolution of amplification products, are of greater interest.

Nested PCR (nPCR) which uses two rounds of amplification, thereby reducing non-specific amplification, is recommended by the WHO/MMV for genotyping and is a technique found frequently in the literature. However it has at least two important limitations. First, it only can detect differences in allelic size, not sequence. Second, amplification products are frequently run on agarose gels; it is widely understood that it is impossible to resolve small differences in the position of bands on such a gel. In appreciation of this, researchers have to decide how to "bin" the results which will determine how close two bands must be in size to be considered a match. Greenhouse *et al.* chose a bin width of 20 base pairs (bp) for the results of their *msp1* and *msp2* analysis (this means that bands within a 20bp range were considered matches)(5); Brockman *et al*. used 40bp bins for *msp1* and *msp2* and 60bp bins for *glurp*.(28)

Heteroduplex tracking assays (HTAs), which use radiolabeled probes to bind to host amplicons, are more sensitive to size differences than PCR and are able to detect insertions, deletions, and clustered base-pair mismatches.(21, 29) Additionally, PCR

appears unable to detect minority populations of parasites, those that make-up less than 10 to 20% of the total within-host population.(30, 31) Using HTAs, one study found that five of six new infections (83%) identified by PCR-correction were actually true recrudescences.(29) This was likely the result of PCR insensitivity to minority variants.(29) Because HTAs use radioactive probes they are not available for use in much of the developing world, however a new HTA that uses a non-radioactive, chemiluminescent probe appears effective.(32) The insensitivity of PCR to minority variants is troubling; when minority variants in the pre-treatment sample are not detected it can lead to misclassification of recrudescent infections which artificially inflates estimates of drug efficacy.

3. Biological factors that complicate PCR-correction

a. Gametocytes

Gametocytes are the sexual stage of malaria parasites and do not replicate or cause disease within a human host. They circulate in the peripheral blood and are ingested by mosquitoes during feeding resulting in parasite transmission. Gametocytes are not susceptible to many antimalarial drugs and have a longer life then other stages of the parasite, living up to 22 days.(2) This is concerning in the context of PCR-correction because they may remain circulating after successful treatment, erroneously reflecting recrudescent parasitemia. Traditional PCR can detect all parasite stages; gametocytes can only be identified differentially using reverse transcriptase-PCR to detect messenger RNA of genes only expressed during this stage. This technique is not widely available and misclassification may occur as a result. Some reassuring evidence was provided by a

study from Uganda that found only 16 of 371 paired samples (4%) had gametocytes, however the methodology they used to identify gametocytes was not reported.(22)

b. Sequestration and synchronicity

Sequestration of parasites and synchronicity may also affect which parasite variants are circulating at detectable levels in the peripheral blood, in turn resulting in incorrect interpretation of genotyping results. In synchronous infections where erythrocytes burst, releasing thousands of merozoite-stage parasites at the same time, parasite densities potentially fluctuate enough to fall below detectable levels.(16) Conversely, asynchronous infections also complicate the interpretation of findings. Snounou and Beck drew attention to this, "many *P. falciparum* infections are relatively asynchronous: the paroxysms can occur at any time, and two or more can be recorded in quick succession, reflecting an intricate dynamic pattern for the growth of different parasite broods."(16) Snounou *et al.* also discuss the potentially misleading role of sequestration of parasites stating that, "late erythrocytic parasite stages are sequestered in the deep vasculature and therefore might not be present in a peripheral sample."(16) Appreciating the potential role of these factors is important although assessing their true impact or adjusting for them in the analysis is beyond the scope of this project.

c. Distribution of alleles in the parasite population

The distribution of alleles in the parasite population impacts the likelihood of observing matching bands in pre- and post-treatment samples. Numerous authors have emphasized that allelic diversity must be high enough to sufficiently reduce the probability that a new infection matches the initial infection by chance.(5, 16, 17, 21, 28, 33) Greenhouse *et al*. used frequency distributions to calculate the probability of a

chance-match (homozygosity) and found values ranging from 0.047 (*msp2*) to 0.18 (TA81, a microsatellite); an increase in allelic diversity reduces homozygosity.(5)

4. Adjustment of PCR-correction through the use of probability theory

PCR-correction is a useful and important tool, though clearly not without limitation. Though impossible to simultaneously address each factor potentially affecting the accuracy of PCR-correction, generating one perfect estimate that truly quantifies drug efficacy, adjustments made based on the allelic distributions of the local parasite population seem an appropriate first step; "In order to optimize the use of PCR genotyping, it is important to calculate the pretest probability of the same genotype occurring in the same individual pre- and posttreatment."(17) These adjustments use probability theory to calculate the probability of a chance-match, i.e. the likelihood of a reinfection matching the pre-treatment sample genotype simply by coincidence.(5, 21, 28, 33) It has been emphasized that patients with multiple infections have a higher probability of a chance-match.(5) The techniques discussed below are a means to adjust for misclassification of new infections; it should be noted that they cannot address misclassification in the other direction (erroneously classifying a recrudescent infection as a new infection because of PCR insensitivity).

Greenhouse *et al*. used a probability-based approach to help answer two questions, (1) how many markers are needed to accurately classify recurrent infections, and (2) how does transmission intensity affect genotyping results?(5) They genotyped 600 pre-treatment and post-treatment samples using *msp2*, *msp1*, and four microsatellites; they also used the pre-treatment samples to generate allele frequency distributions for

each marker. Treating the frequency distributions as probability distributions, they calculated the probability that a pre- and post-treatment allele matched by chance (P_{match}) for each study participant in an area of low to moderate transmission and in a high transmission site. They then adjusted the number of recrudescent infections in each treatment arm separately, as determined by PCR-correction, by the average P_{match} (a thorough description of their approach and their formulas are in Appendix B). They also evaluated the effect of using multiple markers by multiplying the average P_{match} for each marker together to calculate an "overall" P_{match} and adjusted the PCR-corrected results using that value. Their findings confirmed anticipated results: higher allelic diversity conferred a lower P_{match} and higher multiplicity of infection increased P_{match}. The site with lower transmission intensity reached a very low overall P_{match} (0.02) within three markers, whereas the higher transmission site never reached a particularly low P_{match} value even with all six markers (0.16). The authors concluded that in the lower transmission area, "treatment estimates adjusted by genotyping estimates became similar to those adjusted by both genotyping and chance matches… In [the high transmission site], however, the risk estimates remained dissimilar even after genotyping with all six markers... This suggests that even genotyping with the six markers described in this report may substantially overestimate the true risk of treatment failure at very high transmission sites."(5) Overestimating drug failure could result in rejection of a potentially useful compound during drug development.

Kwiek *et al*. evaluated all patients with recurrent parasitemias and calculated how closely they matched (the number of shared bands) and how prevalent the matching alleles were in the parasite population.(21) They considered recurrent parasitemias

sharing a single band that appeared in more than 10% of samples indeterminate (neither a new infection or recrudescence). For indeterminate parasitemias, they then used the prevalence of the shared allele and the number of variants in the recurrent sample to calculate an estimated probability of a chance-match. They multiplied the mean probability of treatment failure (1 – the mean of the chance-match probabilities) by the number of indeterminate infections and adjusted the proportion of treatment failures accordingly (Formula in Appendix C). Like Greenhouse *et al*., they concluded that probability-based adjustments of genotype-corrected rates (they used HTAs, not PCR) may be useful in high transmission areas.(21)

Not all researchers agree that chance-matches play an important role.(18, 23) However, the findings of studies that used probability-based adjustments of genotyped results(5, 21, 28, 33) strongly indicate that this is incorrect.

5. Uncertainty analyses

Traditional estimation of confidence intervals accounts only for random error. Bayesian methods can be used to incorporate prior information on biases and other methods have also been developed.(34-37) An analysis presented by Jurek *et al.* corrected for outcome misclassification using a Monte Carlo analysis instead of a Bayesian analysis. They argued this was sufficient because they were not specifying a prior distribution of the parameter of interest itself, only prior distributions related to the misclassification.(36) Though their subject area was not infectious diseases, modifications to their approach would make it relevant to outcome misclassification in antimalarial efficacy trials.

In conclusion, PCR-correction is a useful first step in estimating treatment efficacy, but without the incorporation of additional information, such as the distribution of genetic variants in the parasite population and multiplicity of infection, it can result in misclassification of a patient's outcome. The ability of PCR-correction to correctly classify a patient's outcome is also limited by the insensitivity of PCR to minority variants. The use of probability-based adjustments, can take these factors into account, could potentially play an important role in generating more reliable estimates of cure rates.

B. The effect of HIV-1 protease inhibitors on incidence of malaria

Protease inhibitors are not currently recommended for use as first-line ART in Sub-Saharan Africa.(38) However, the recent advent of a heat stable co-formulation of two PIs, lopinavir and ritonavir (LPV/r), coupled with the demonstrated resistance to first-line non-nucleoside reverse transcriptase inhibitor (NNRTI)-based therapy(39) makes it likely the use of PIs will increase. Furthermore, laboratory evidence has shown that LPV/r and other HIV PIs inhibit the growth of *P. falciparum*; if this is found to also be true in humans, the use of HIV PIs in malaria-endemic parts of the world would be even more valuable.

The Adult AIDS Clinical Trials Group (AACTG) conducted a study comprising two phase III randomized clinical trials (RCT): one for HIV-1-infected, treatment-naïve women and one for HIV-1-infected women who have been exposed

to single-dose nevirapine (NVP) to prevent mother-to-child transmission of HIV-1. In each RCT there were two treatment arms; one in which patients received NNRTI-based therapy, the other in which patients received protease inhibitor LPV/r-based therapy.(40) We conducted an ancillary study using data collected by the AACTG trials to look at the effect of LPV/r on *P. falciparum* infection in adults.

1. Malaria and HIV-1

The geographical overlap of malaria and HIV is striking. It is estimated that almost one million people die of malaria every year; most of whom live in Sub-Saharan Africa.(1) The Joint United Nations Programme on HIV/AIDS reported that roughly 22.5 million people were living with HIV/AIDS in Sub-Saharan Africa in 2007; a far greater number than any other region of the world.(41)

The biological interaction between HIV-1 and malaria is well documented (all references to HIV for the remainder of the document refer to HIV-1). The reciprocal nature of the interaction – HIV increases malaria incidence and worsens clinical manifestations, malaria elevates HIV viral load – makes it crucial to understand its repercussions in an effort to improve prevention and treatment strategies. Using a mathematical model, Abu-Raddad *et al.* estimated that in an adult population of approximately 200,000, and in an area in which both HIV and malaria are highly prevalent and malaria interventions not used, between 1980 and 2006 the interaction may have led to more than 8,000 excess cases of HIV and almost one million excess cases of malaria.(42)

a. The effect of HIV on malaria

HIV is associated with increased parasitemia, clinical malaria and severe malaria. In 2000, Whitworth *et al.* described the role of HIV on parasitemia and episodes of clinical malaria.(43) Based in a malaria-endemic region of Uganda, the study followed a cohort of 484 adults from 1990-1998 and conducted both scheduled and interim visits when participants felt ill. HIV was associated with increased odds of both parasitemia and clinical malaria (Table 1). Among HIV-infected individuals, those with lower CD4 counts tended to have higher parasite burdens.(43)

Table 1. Malaria status among study participants at routinely scheduled visits

HIV status	Parasitemia N/total (%)	Odds ratio (95% CI)*	Clinical Malaria N/total (%)	Odds ratio (95% CI)*
Negative	231/3688 (6.3)	-	26/3688 (0.7)	-
Positive	328/2788 (11.8)	1.81 (1.43, 2.29)	55/2788 (2.0)	2.56 (1.53, 4.29)

Data taken from Whitworth *et al.(43)* *Odds ratios adjusted for age, sex and pregnancy

Patnaik *et al*. evaluated the effect of HIV serostatus, viral load, and CD4 counts on parasitemia. They calculated hazard ratios (HR) and found that first-episode, second-episode and overall incidence of parasitemia were all greater in HIV-infected adult study participants who were followed for a single rainy season in Malawi, a malaria-endemic country.(44) They found an increased rate of first-episode parasitemia associated with increased HIV RNA concentration (Adjusted HR (95% CI) per 1-log increase = 1.24

(1.02, 1.51)) and of second-episode parasitemia (Adjusted HR (95% CI) per 1-log increase = 2.12 (1.14, 3.92)). The direction of this association was the same when considering overall incidence of parasitemia though not statistically significant (Adjusted HR (95% CI) per 1-log increase = 1.24 (1.02, 1.51)). (It is of note that when assessing the effect of HIV RNA concentration they did not adjust for CD4 count.) The hazard of a first-episode of malaria was lowest among individuals with ≥ 400 CD4 cells/μl.(44)

Clinical malaria is also associated with HIV and its resulting immunosuppression. Evidence suggests that there is an increased relative risk of clinical malaria among individuals with fewer CD4 cells and more advanced HIV disease.(43) In a cohort of HIV-infected adults in Uganda, the rate of malarial febrile episodes among individuals with fewer than 200 CD4 cells/μl was more than twice that of the rate among individuals with > 500 CD4 cells/μl (139.8/1000 person-years compared to 57.3/1000 person-years).(45)

Grimwade *et al*. conducted a study in an area of unstable malaria transmission to observe the effect of co-infection among a population with lower levels of the partial-immunity one would expect to find in endemic regions.(46) They reported a significant association between HIV and severe malaria disease among adults with confirmed malaria [Adjusted Odds Ratio (OR) (95% CI) 2.3(1.4, 3.9)].(46)

In summary, HIV has been linked to increased parasitemia, clinical malaria, and the severity of malaria disease among adults. This association is found in both endemic areas and areas of unstable transmission.

b. The effect of malaria on HIV

Malaria also exacerbates HIV infection. Kublin *et al*. conducted a prospective cohort study that followed HIV-infected patients at regularly scheduled and interim visits when participants experienced illness.(47) They measured the amount of HIV RNA at baseline, at the time a patient was found to be parasitemic and, on average, 8-9 weeks post-malaria. They found that concentration of viral RNA, for patients with baseline CD4 >300/µl, nearly doubled during the episode of malaria; this effect was even stronger among patients with high levels of parasitemia (≥2000/µl) and fever. They observed no significant difference in HIV RNA levels over time for participants who did not experience an episode of parasitemia.(47) In another cohort of HIV-infected individuals, patients with clinical malaria had a median viral load almost seven times that of non-parasitemic controls.(48) Though the effect of high viral load may in fact have made the patient more susceptible to malaria (thereby confusing this effect), there was a progressive decline of median viral load after treatment for malaria and after four weeks there was no significant difference in median viral load between individuals recovering from malaria and the control group.(48) The mechanism by which malaria increases viral load is still under study; some evidence suggests it may be related to increased production of TNF-α.(49, 50)

Malaria is also associated with a decline in CD4 cells.(51) After adjusting for baseline variables, including baseline CD4 count, Mermin *et al*. reported that the average decline in CD4 cells was, per episode of malaria, 40.5/µl. When compared to HIV-infected individuals who experienced no episodes of malaria, people who had at least three episodes experienced an annual decrease in CD4 cells of more than 140/µl.(51)

2. The treatment of HIV-1 in Sub-Saharan Africa

In the 2006 antiretroviral treatment (ART) guidelines for resource-poor countries issued by the WHO, a combination of three drugs was recommended: two nucleoside reverse transcriptase inhibitors (NRTI) and a single NNRTI (either efavirenz (EFV) or NVP).(52) NVP is less expensive than EFV and is widely used, however its use carries with it risk of severe rash and rarely hepatotoxicity.(52)

The 2009 revisions to the WHO guidelines do not recommend PI-based therapy as a first-line treatment, only a second-line treatment.(38) However the new availability of heat stable LPV/r in combination with observation that resistance to the complete class of NNRTI therapies can result from a single nucleotide polymorphism in HIV-1(52) makes reassessment of PI-based therapy important. One aim of the AACTG study was to investigate the possibility that NNRTI-based ART is less effective in patients with previous NNRTI exposure (in the form of single-dose NVP) due to the selection of resistant virus resulting from that exposure. This possibility makes the option of using PI-based therapy as a first-line treatment attractive and worthy of further consideration. Second line therapy use in the developing world is also increasing over time and is almost exclusively PI-based.

3. Diagnosis and treatment of co-infected individuals

There are risks associated with concurrent use of antimalarials and antiretrovirals.(53, 54) Brentlinger *et al.* reviewed the diagnostic and treatment challenges resulting from the overlap of the two infections, highlighting the need for medications effective against both diseases.(53) They proposed five reasons why “the HIV-infected patient residing where malaria is endemic or epidemic may be at risk of

misdiagnosis and mismanagement…" In summary they are: 1) symptoms of both conditions can be varied "[making] clinical decision making…difficult"; 2) malaria may occur simulatneously with other infections or even adverse reactions to ART, causing difficulties for patient management; 3) a possible increase in unneeded malaria treatment in patients with fever, or alternatively failing to diagnosis malaria in a patient with symptoms associated with HIV or adverse reactions to ART; 4) insufficient information available for evidence-based concomitant treatment of both infections; and, 5) lack of clinical facilities to correctly diagnose malaria. The "overlapping adverse effect profiles" of certain antiretroviral and antimalarial drugs, and the known deliterious drug interactions between the two types of treatments(53), make the possability of a medication that can treat both diseases highly desirable.

There are drugs used as chemoprophylaxis in HIV-infected patients that protect them from malaria. For example, co-trimoxazole use in HIV-infected patients reduces incidence of malaria.(55). Though co-trimoxazole reduces morbidity and mortality among those infected with HIV, it is does not treat or cure HIV.

4. Antimalarial action of protease inhibitors

The antimalarial properties of PIs were first demonstrated in laboratory studies more than a decade ago. In Rosenthal's 1995 article in *Experimental Parasitology*, he reported the deleterious effect of PIs on the malaria parasite.(56) Malaria parasites construct proteins by hydrolyzing the host erythrocyte's hemoglobin and using the resulting amino acids.(57) Rosenthal cultured parasites with different chemical agents and found that cysteine PIs caused morphological changes in the food vacuole of the

parasite.(56) Aspartic PIs did not cause such an abnormality but were also toxic to the parasite.(56) This *in vitro* evidence warrented further investigation into the antimalarial nature of PIs.

Experimental approaches to quantifying antimalarial effects relevant to this dissertation have primarily involved incubating cultured parasites, of various antimalarial drug sensitivities, with HIV PIs and measuring growth inhibition. Skinner-Adams *et al.* published the "first report that antiretroviral PIs can directly inhibit *in vitro* growth of both drug-sensitive and drug-resistant *P. falciparum* parasites."(58) The observed efficacy against drug-resistant parasites is particularly important in the context of growing antimalarial drug-resistance. Several agents were particularly harmful to the growth of the parasite including RTV, a component of LPV/r, whereas NVP had no effect.(58) Growth inhibition resulting from exposure to concentrations of LPV (0.9-2.1μM) which are lower than those found in the plasma of a patient on LPV/r ART have also been described.(59) It is of note, however, that LPV/r is 98-99% protein bound(60); this may indicate that the *in vitro* concentrations resulting in parasite growth inhibition may be higher than those freely available in a patient. Parasites exposed to LPV alone, RTV alone, or LPV/r experienced growth inhibition and exposure to RTV alone resulted in morphological changes of the parasites.(61) In an *ex vivo* experiment, parasites exposed to sera taken from HIV-infected patients taking LPV/r had a 50-95% reduction in growth when compared to serum from controls.(62)

Andrews *et al.* provided the first evidence of an *in vivo* effect of LPV/r.(61) They compared the efficacy of multiple chemotherapeutic agents against the non-lethal murine model of malaria, *P. chabaudi*. LPV/r decreased the median peak parasitemia and

delayed onset of parasitemia by two days (compared to the control).(61) Evidence from a different murine model, *P. yoelii* suggested that HIV PIs, including LPV, also demonstrate action against pre-erythrocytic stage parasites.(63)

The mechanism for the observed antimalarial effect of HIV PIs is still unknown; initially it was believed that the agents interfered with plasmepsins I-IV (four of the ten *P. falciparum* aspartyl proteases) which function within the food vacuole.(58) Additional evidence bolstered this hypothesis: structural similarities between plasmepsin II and the HIV protease,(64) docking studies that suggested bonding interactions between HIV PIs and plasmepsins II and IV are possible,(61) and by measuring the inhibition of a "hemoglobin-based peptide substrate by recombinant plasmepsin II," it was found that plasmepsin II was inhibited by LPV and RTV *in vitro*.(59)

New evidence, however, suggests that the antimalarial effect of HIV PIs may not be related to food vacuole plasmepsins. In both drug interaction studies and experiments with knockout parasites, Parikh *et al*. concluded that HIV PIs do not act in the same way as pepstatin, an aspartic protease inhibitor known to have antimalarial action.(65) Experiments that measure the interaction of HIV PIs and chloroquine also support a non-food vacuole mechanism of action. The antimalarial action of chloroquine is not fully understood but it is believed to act on heme, the iron-containing prosthetic group of hemoglobin, after it is cleaved from the hemoglobin molecule by the food vacuole plasmepsins.(66) Synergism between HIV PIs and chloroquine would therefore be unexpected if HIV PIs inhibit food vacuole plasmepsins. However, synergism does occur.(67, 68) Additionally, RTV and saquinavir (another HIV PI), suppress growth of *P. vivax*.(69) *P. vivax* has only a single ortholog to a *P. falciparum* food vacuole

plasmepsin(70), and that ortholog is not transcribed during the ring stage, suggesting that the food vacuole plasmepsins are not the target of HIV PIs.(69) Recent experiments that explored antimalarial activity throughout the parasite's within-human life cycle, found that HIV PIs were responsible for the strongest growth inhibition during the trophozoite and schizont stages and also acted on gametocytes, leading the researchers to cautiously conclude, "that the primary target of the PIs is likely to be expressed in both gametocytes and intra-erythrocytic parasites…plasmepsins V, IX, and X appear to be the best candidate targets of these drugs."(71)

Nathoo *et al.* proposed that HIV PIs may have a beneficial consequence for patients with malaria independent of antiparasitic effects.(72) *In vitro,* they found a marked reduction in the expression of CD36, a human surface receptor associated with the binding of malaria parasites, after exposure to several ART compounds, including RTV. They observed that the "induced CD36 deficiency [results] in decreased CD36-mediated cytoadherence…of parasitised erythrocytes." The authors did, however, caution that decreased expression of CD36 could also potentially harm the patient, postulating that parasites may then simply bind to another surface receptor, ICAM-1, which is implicated in cerebral malaria. Additionally, the decrease in CD36 also reduced phagocytosis of parasitized erythrocytes which may reduce the patient's ability to fight the infection.(72)

In conclusion, though laboratory evidence supports the antimalarial effect of HIV PIs on malaria parasites, the mechanism for that action remains unknown. Additionally, HIV PIs may reduce the parasite's ability to bind to host cells though the repercussions of this effect are not understood. Currently there is nothing known about an antimalarial

effect of HIV PIs in humans. Data from the AACTG trials provide the first opportunity to examine incidence of malaria among HIV-infected individuals on PI-based therapy.

CHAPTER 2

RESEARCH DESIGN AND METHODS

A. Factors influencing PCR-corrected cure rates in antimalarial efficacy trials

This aim had two objectives. First, to demonstrate the effect of the distribution of allelic variants, transmission intensity and MOI on the probability of misclassification of recurrent infections. Second, to develop a practical approach for adjusting PCR-corrected results for misclassification of both reinfections and recrudescences, and provide a worked example using data from areas of both high and low transmission intensity.

1. Characteristics affecting the probability of false positives

We used simulations of the infection, cure, and re-infection process to demonstrate the effect of allelic diversity, transmission intensity and MOI on the probability of a false positive. In this context, a false positive refers to a reinfection that is misclassified as a recrudescence because allelic variants in the day 0 and day R samples match by chance. We used MATLAB R2008a (Natick, MA) software to simulate infections (and re-infections) of individual patients after specifying the population-wide distribution of allelic variants. The parameters of these distributions

were based on values in the literature on relevant *P. falciparum* genetic markers. For each of 100,000 simulated patients, we assigned a specified number of day 0 variants drawn randomly from the distribution. We set treatment success at 100% and assigned a specified number of day R variants the same way. We tested all patients for matching day 0 and day R variants, and calculated the probability of a false positive as the number of patients with a match divided by 100,000, the number of simulated patients.

We first assessed the effect of allelic diversity in the parasite population on the probability of a false positive. As in routine PCR-correction, allelic variants were distinguished by the number of base pairs (bp); due to the insensitivity of nPCR to small variations in the number of bp, variants that were different by no more than 20bp were considered to be the same to replicate the degree of precision routinely allowed. We used allelic distributions appearing in the literature to inform a plausible mean, 350bp, and a wide range of variances, from 1575 to 6475, to generate ten negative binomial distributions. The negative binomial distribution is believed to most accurately represent allelic distributions within parasite populations.(73) For each distribution, we simulated the infection and reinfection of 100,000 patients by assigning each a single day 0 variant and a single day R variant drawn randomly from the distribution.

We assessed the effect of transmission intensity on the probability of a false positive by assigning each patient one day 0 variant and one, two, three or four day R variants, each reflecting an infectious bite (for simplicity, we assumed each infectious bite transmitted a single variant). We simulated the effect of MOI similarly, assigning each patient one through four day 0 variants and the same number of day R variants.

2. Monte Carlo uncertainty analysis

a. Theory

To accurately measure treatment success, estimates of the cure rate need to be adjusted for two types of misclassification: false positives (reinfections incorrectly being classified as recrudescent) and false negatives (true recrudescent infections misclassified as reinfections because a minority variant in the day 0 sample was not detected by nPCR). To adjust for this misclassification, we developed an uncertainty analysis that requires two sources of external, or prior, information: the distributions of false positives and false negatives. These distributions can be estimated using data from antimalarial efficacy studies.

We developed a method for estimating the distribution of false positives that reflects our understanding of the factors that influence the probability of a chance match and exploits characteristics of the study data themselves, allowing the probability of a false positive to appropriately be tailored to the study setting. False positive probabilities were calculated using the same simulation procedure described above, except that the number of allelic variants observed in each patient at day 0 and day R, and the population-wide distribution of allelic variants were set to match study data. We used MATLAB R2008a (Natick, MA) to simulate the infection and reinfection of N patients, where N was the number of patients who participated in the study. Each patient was assigned X day 0 variants and Y day R variants from the day 0 and day R distributions of allelic variants generated by genotyping parasites present in study samples (the X for each patient was randomly selected from the observed distribution of the number of day 0 infections, the Y randomly selected from the distribution of the number of day R

infections) and tested for matches. The false positive probability for this simulated study was then calculated as the number of chance matches divided by N. We repeated this process 10,000 times (generating 10,000 false positive probabilities) to create the distribution of the proportion of recrudescent infections that were false positives.

To estimate the distribution of false negatives, we made use of the observation that nPCR has limited sensitivity to variants comprising less than 20% of a patient's parasite population.(30, 31) Misclassification of a recrudescence as a reinfection, a false negative, requires that each day R variant be undetected in the day 0 variants, as a single shared variant will result in the classification of the recurrence as a recrudescence according to the MMV/WHO guidelines.(20) To our knowledge, the only published information on the role of false negatives comes from Juliano *et al.* who used heteroduplex tracking assays (HTAs), a molecular method more sensitive to minority variants and genetic variation than nPCR, and found that five of six new infections (83%) identified by PCR-correction were truly recrudescent infections.(29) However, their study population was at negligible risk of reinfection, likely making their results an overestimate in the context of an average antimalarial trial. Therefore, to estimate the proportion of reinfections that were false negatives we used the median number of variants observed in the day R samples, assumed each variant carried with it a 20% chance of being missed in the day 0 sample, and calculated the probability that all were missed at day 0 resulting in a false negative using this formula: proportion of false negatives equals $(0.2)^v$, where v is the median number of variants. The 20% chance was based on existing literature and expert opinion. We also conducted a sensitivity analysis varying the probability of a band being missed in the day 0 sample from 0 to 80%.

We conducted a Monte Carlo uncertainty analysis to adjust the observed number of recrudescent infections as determined by PCR-correction after genotyping *msp2* by the estimated distributions of false positives and false negatives. Using an approach similar to that described by Jurek *et al*.,(36) we calculate the adjusted cure rate using this formula:

$$\text{Adjusted cure rate} = [N_t - (N_{recru} - (N_{recru} \times FP) + (N_{new} \times FN))] / N_t \quad (1)$$

Where N_t is the total number of patients, N_{recru} is the number of recrudescent infections identified by PCR-correction, FP is the proportion of recrudescent infections that were false positives, N_{new} is the number of reinfections identified by PCR-correction, and FN is the proportion of reinfections that were false negatives.

We used Oracle Crystal Ball, Fusion Edition (Redwood Shores, CA) software to run 100,000 trials in which the number of recrudescent infections as determined by PCR-correction after genotyping *msp2* in each study area was adjusted and the cure rate calculated using formula 1 (above). As the last step in each trial, we included a bootstrap step to allow for sampling error by generating a random value from a binomial distribution in which the number of trials was equal to the number of patients in the study and the probability of success was the uncertainty-adjusted probability of treatment failure. This approach is appropriate for estimating the sampling error of an estimated proportion.(74) These random values were then used as the number of recrudescences to calculate the final cure rate, adjusted for both uncertainty and sampling error. We also ran 100,000 trials without the bootstrap step to explore the effect of uncertainty in the

absence of sampling error, and finally, also calculated traditional 95% confidence intervals around the PCR-corrected cure rate with no adjustment for outcome misclassification to demonstrate the effect of sampling error in the absence of uncertainty about the outcome.

b. Example data

To provide an example of our proposed uncertainty analysis, we used genotyping data from two randomized antimalarial efficacy trials conducted in areas of differing transmission intensity. The data from the high transmission area came from a study in Tororo, Uganda (N=401); the researchers were comparing the efficacy of an amodiaquine plus artesunate regimen compared to an atemether-lumefantrine regimen.(4) The data from the low transmission area were generated by a study conducted in Bobo-Dioulasso, Burkina Faso (N=827); the researchers were comparing the efficacy of amodiaquine, sulfadoxine-pyrimethamine and amodiaquine plus sulfadoxine-pyrimethamine.(75) In both studies, the different therapies did demonstrate different levels of efficacy(4, 75); however, because we are not interested in a particular treatment's efficacy, and instead are simply providing an example of the uncertainty analysis, we did not stratify by treatment arm. The data for each patient included the number and identity of allelic variants. Greenhouse *et al*. used two sets of primers for amplification to capture two allelic families of *msp2*, IC3D7 and FC27.(5) To prevent artificial chance-matches (a variant amplified with one set of primers that was 300bp is not the same as a variant of 300bp amplified with the other set of primers), we added 1000bp to each variant identified by the FC27 primers to create a single distribution of *msp2* variants that included both allelic families.

B. The effect of HIV-1 protease inhibitors on incidence of malaria

The AACTG conducted two phase III randomized clinical trials (ACTG5208) to measure HIV outcomes for HIV-infected women with and without previous NVP exposure when randomized to either LPV/r-based therapy or NNRTI-based therapy. We conducted an ancillary study using the AACTG data to measure the association between LPV/r and clinical malaria in humans.

1. ACTG5208 and selection of participants for ancillary study

The AACTG screened and recruited HIV-infected, treatment-naïve women for participation in one of the two trials of which the parent-study was comprised.(40) One trial enrolled women with no history of NVP exposure (N=502); the other trial enrolled women who had received single-dose NVP to prevent mother-to-child transmission of HIV(N=243).(40) Once it was determined in which trial a participant was to be placed, she was randomly assigned (1:1) to a treatment arm.(40) Participants were followed until 48 after weeks after the final participant was randomized.(40)

In both trials, women were randomized to receive either LPV/r-based therapy or NNRTI-based therapy. As a part of their therapy, all participants received two nucleoside reverse transcriptase inhibitors (NRTI) consistent with WHO treatment guidelines.(52) The parent-study recommended emtricitabine and tenofovir; however clinicians at sites were encouraged to select the NRTIs used as he/she deemed most appropriate.(40)

The AACTG enrolled women at least 13 years of age (or older as dictated by the study site IRB) with a CD4+ cell count < 200 cells/mm^3 obtained within 90 days prior to study entry. Additional biological parameters required for enrollment including:

- Absolute neutrophil count ≥ 750/mm^3
- Hemoglobin ≥ 7.0 g/dL
- Platelet count ≥ 50,000/mm^3
- Total bilirubin ≤ 2.5 x upper limits of normal
- Aspartate aminotransferase, Alanine aminotransferase, and alkaline phosphatase ≤ 2.5 x upper limits of normal
- Negative pregnancy test within 45 days prior to study entry

The study population was also subject to the following conditions:

- All women who could potentially become pregnant must have agreed to use birth control for the duration of the study and for six weeks following the discontinuation of study medication.
- All participants must have had a Karnofsky performance score ≥ 70 within 45 days prior to study entry. (A Karnofsky performance score characterizes functional impairment.)
- Ability/willingness of participant (or legal guardian/representative) to give informed consent.
- Intent to remain in current geographical area of residence and attend study visits as required.

The study population did not include women confined in a correctional facility for legal reasons or in a medical facility for treatment of either a psychiatric or physical illness.

We used data from participants in both trials from all of the malaria-endemic study sites: Eldoret and Kericho, Kenya; Lilongwe, Malawi; Kampala, Uganda; Lusaka, Zambia; and Harare, Zimbabwe.

2. Study measures

a. Main exposure

The main exposure was the therapeutic regimen to which the participant was randomized: LPV/r-based therapy or NNRTI-based therapy. Participants receiving LPV/r were considered exposed; participants receiving an NNRTI were the referent group. LPV/r is a co-formulation of two aspartic protease inhibitors used as part of antiretroviral regimens. When used in combination, the RTV increases availability of LPV in the patient (the efficacy and tolerability of this combination of these drugs is reviewed in (76). Aspartic protease inhibitors have exhibited antimalarial properties in multiple studies.(58, 59, 61-63, 71) The NNRTI used in the study was NVP. NVP is a widely used therapeutic agent, used in both NNRTI-based ART and as a single-dose treatment to prevent mother-to-child transmission. NVP has not demonstrated antimalarial action.(58, 62)

b. Outcomes

We used two definitions of malaria: confirmed malaria and probable malaria (Table 2). These definitions were developed by the trial staff. Confirmed malaria

required both identification of *Plasmodium* sp. on a peripheral blood smear and "compatible clinical syndrome." (ACTG materials) Probable malaria required both a "compatible clinical syndrome" and antimalarial treatment employed or recommended. (ACTG material) We also included patients who were prescribed antimalarials (amodiaquine, artemether/lumefantrine, artesunate, chloroquine phosphate, dihydroartemisinin/piperaquine, mefloquine HCl, pyrimethamine/sulfadoxine, quinine dihydrochloride, quinine sulfate or sulphalene/pyrimethamine) without a recorded diagnosis as probable cases.

Peripheral blood smears are used to identify parasites with microscopy and is considered the gold standard in malaria diagnosis. Limitations to the approach include: low parasite densities may go undetected, variability in how samples are processed may lead to misdiagnosis and the microscopist must be highly trained.(77) An additional limitation in the context of this study was that the parent-study did not regularly collect blood smears as part of the protocol; instead they collected them when testing for malaria was appropriate based on the trial site's standard of care guidelines. Data describing the magnitude of participants' parasitemia were unavailable.

c. Covariates: The use of an intent-to-treat analysis simplifies our analysis as theoretically it controls for both measured and unmeasured confounders. We stratified by trial and study site.

Use of concomitant medications with antimalarial activity, such as cotrimoxaole, could potentially modify the effect of the main exposure on the incidence of malaria. Seasonal variation in malaria transmission intensity could also

modify that relationship. We used product terms in the models to evaluate the impact of these two possible effect measure modifiers. Using climate data from the National Oceanic and Atmospheric Administration and evidence from the literature(78-82), we created a dichotomous time-varying variable denoting rainy season (indicating a higher risk of malaria transmission). We also created a time-varying dichotomous variable indicating current use of concomitant medications with antimalarial activity (azithromycin, clindamycin, diaminodiphenylsulfone, doxycycline hydrochloride, doxycycline monohydrate or trimethoprim/sulfamethoxazole).

3. Statistical analysis.

We conducted a discrete-time survival analyses (DTSA). This required that the dataset be formatted so that each participant has multiple records, one for each period under observation until she either experienced the event (malaria) or completed follow-up (Appendix E). We ran two final models: one in which both probable and confirmed cases were considered events and, as a sensitivity analysis, one in which only confirmed cases were considered events. All analyses were intent-to-treat.

a. Rationale and interpretation

DTSA uses hazard functions to quantify the effect of predictors on event occurrence. Hazard is the proportion of individuals at risk at the beginning of a time period experiencing the event (malaria), conditioned on having not experienced the event in an earlier time period. DTSA affords several advantages. By modeling not only event occurrence but also event timing, we were able to estimate the hazard of malaria across

different time periods, describing any patterns that exist and any difference in hazards between treatment arms over time. Hazards may also be used to calculate survival probabilities, the proportion of the original study population that does not experience the event though successive time periods. Survival analysis is a widely accepted approach with easily interpretable results.

The parameters estimated by DTSA hazard models are simple to interpret (see Appendix F for the formula of the DTSA model); the alpha coefficients correspond to time period-specific baseline hazards (the hazards of the reference group), and the beta coefficients, when exponentiated, are the hazard ratio associated with a one unit change in the predictor in any time period. (We used a pooled logistic regression model in which the exponentiated beta is a good estimate of the hazard ratio as long as the event proportion in all discrete time periods is less than 10%.) In addition to presenting our estimated parameters and hazard ratios, we plotted logit hazards and survival probabilities to visualize the effect of therapeutic regiment on the hazard of malaria.

b. Assumptions

Survival analyses include information from both censored and non-censored participants; this assumes that censoring is independent of event occurrence. There are three key assumptions underlying the use of the discrete-time hazard model:

1. Proportionality. As in continuous-time survival analyses, the assumption of proportionality requires that the effect of a predictor does not vary across time; in the discrete-time survival analysis, this means that all of the logit-hazard profiles resulting from stratification by a predictor will have the same shape and be equidistant across all time periods. It should be noted, however, that an interaction between time and another

predictor has been described as “the rule, rather than the exception.”(83) Fortunately, a violation of this nature can be identified (and resolved) by including product terms (predictors*time) in the model and evaluating model fit.

2. Linear additivity. The discrete-time hazard model requires that one unit changes in the value of a predictor all have the same effect on the logit hazard. One way this assumption can easily be tested is stratifying by predictor values, calculating the logit-hazard at each level of the predictor and plotting the values. If single unit changes in the predictor generate equal displacements of the logit hazard, the assumption is met. Transformation or categorization of predictors that violate this assumption may achieve linearity. On the logit scale, the combined effect of predictors is assumed to be additive (i.e. no statistical interaction). This assumption will be explored by plotting stratified sample hazard functions and changes in model fit when interaction terms are included.

3. No unmeasured heterogeneity. “All variation in hazard profiles across individuals is hypothesized to depend solely on observed variation in the predictors.”(83) Unlike the other assumptions, violations of this assumption are not easily identified or rectified and the inclusion of an error term appears to be problematic. However, because our data come from RCTs we do not anticipate that our models violated this assumption.

c. Model-building

The same model-building strategy was employed for both models (one in which either definition of malaria was considered an event, and when only confirmed cases were considered events). The independent variable is the event indicator, a dichotomous variable that appears in each record of all participants. We first created a model to assess the effect of time only. We had weekly data with follow-up times

up to 144 weeks; creating indicator variables for each week, as is frequently done in DTSA, was inappropriate. We explored multiple representations of time including: linear, quadratic, cubic, higher order polynomials, logarithmic transformations and restricted cubic splines using Harrell's DASPLINE SAS macro.(84) We used goodness of fit statistics to determine which representation of time improved model fit sufficiently to justify the reduction in parsimony. Using product terms, we also explored whether the effect of therapy varied over time (if so, it would violate the proportionality assumption and the terms would be required in the model).

Next we added our dichotomous exposure to the model (LPV/r-based therapy or NNRTI-based therapy). We then added trial, study site, and other variables of interest into the model, one at a time. Traditional model diagnostics such as deviance statistics and information criteria were employed to evaluate the benefit of adding these terms to the model. We assessed effect measure modification through the inclusion of product terms and quantified their effect on the overall fit of the model using deviance statistics.

CHAPTER 3

ADJUSTING FOR MISCLASSIFICATION IN ANTIMLALARIAL EFFICACY STUDIES

A. Summary

Evaluation of antimalarial efficacy is difficult because recurrent parasitemia can be due to recrudescence (drug failure) or reinfection. PCR is used to differentiate between recrudescences and reinfections by comparing parasite allelic variants before and after treatment. However, PCR-corrected results are susceptible to misclassification: false recrudescences, due to reinfection by the same variant present in the patient before treatment; and false reinfections, due to variants that are present but too infrequent to be detected in the pre-treatment PCR, but are then detectable post-treatment. We explored factors affecting the probability of false recrudescences and proposed a Monte Carlo uncertainty analysis to adjust for both types of misclassification. Higher levels of transmission intensity, increased multiplicity of infection, and limited allelic variation resulted in more false recrudescences. The uncertainty analysis exploits characteristics of study data to minimize bias in the estimate of efficacy and can be applied to areas of different transmission intensity.

B. Introduction

The World Health Organization (WHO) recommends that first-line antimalarial treatment policies be changed when a drug's cure rate falls below 90%, and that new treatments not be recommended unless they have a cure rate greater than 95%.(14) However, defining the antimalarial cure rate is difficult in falciparum malaria clinical trials because recurrent parasitemias can result from either recrudescence (drug failure) or reinfection during follow-up.

One tool used to distinguish between reinfection and recrudescence is PCR-correction (or PCR-adjustment). PCR-correction most often uses nested PCR (nPCR) to categorize recurrences by comparing size polymorphisms in genetic markers [merozoite surface proteins 1 and 2 (*msp1, msp2*) and glutamate rich protein *(glurp*)] before and after treatment. PCR-correction of cure rates has been in use for more than 20 years and there is an extensive literature on the substantial impact it can have on estimates of treatment efficacy, as previously reviewed.(15, 17) Variations in PCR-correction techniques exist, especially with regard to the interpretation of results. In response to this variability, the Medicines for Malaria Venture (MMV) collaborated with the WHO to generate guidelines for PCR-correction including a definition of a recrudescent infection, namely a recurrence in which one or more allelic variants are shared in the pre-treatment (day 0) sample and the recurrent (day R) parasitemia.(20)

PCR-correction is fallible. Incorrect identification of a reinfection as a recrudescence occurs when the patient is infected with same variant before and after treatment; this is more likely to occur in an area with limited allelic diversity or high

transmission intensity.(5, 21) This type of misclassification results in underestimation of the cure rate. Additionally, there are often multiple genetically distinct allelic variants present within a single host and nPCR is not capable of detecting minority variants representing <20% of the population.(85) Thus, PCR-correction could misclassify a recrudescence as a reinfection because an apparently "new" variant which appears in the day R sample was present, but not detected, in the day 0 sample.(29) This may be particularly important if drug resistant variants are at levels below detection initially but become more prevalent in the patient as other variants are cleared by the treatment. This type of misclassification results in overestimation of the cure rate.

This present work has two aims. First, to demonstrate the effect of the distribution of allelic variants, transmission intensity and multiplicity of infection (MOI) on the probability of misclassification of recurrent infections. Second, to develop a practical approach for adjusting PCR-corrected results for misclassification of both reinfections and recrudescences. A worked example using data from areas of both high and low transmission intensity is provided.

C. Methods

1. Characteristics affecting the probability of false positives

We used simulations of the infection, cure, and re-infection process to demonstrate the effect of allelic diversity, transmission intensity and MOI on the probability of a false positive. In this context, a false positive refers to a reinfection that is misclassified as a recrudescence because allelic variants in the day 0 and day R

samples match by chance. We used MATLAB R2008a (Natick, MA) software to simulate infections (and re-infections) of individual patients after specifying the population-wide distribution of allelic variants. For each of 100,000 simulated patients, we assigned a specified number of day 0 variants drawn randomly from the distribution. We set treatment success at 100% and assigned a specified number of day R variants the same way. We tested all patients for matching day 0 and day R variants, and calculated the probability of a false positive as the number of patients with a match divided by 100,000, the number of simulated patients.

We first assessed the effect of allelic diversity in the parasite population on the probability of a false positive. As in routine PCR-correction, allelic variants were distinguished by the number of base pairs (bp); due to the insensitivity of nPCR to small variations in the number of bp, variants that were different by no more than 20bp were considered to be the same to replicate the degree of precision routinely allowed. We used allelic distributions appearing in the literature to inform a plausible mean, 350bp, and a wide range of variances, from 1575 to 6475, to generate ten negative binomial distributions, shown in Figure 4. The negative binomial distribution is believed to most accurately represent allelic distributions within parasite populations.(73) For each distribution, we simulated the infection and reinfection of 100,000 patients by assigning each a single day 0 variant and a single day R variant drawn randomly from the distribution.

We assessed the effect of transmission intensity on the probability of a false positive by assigning each patient one day 0 variant and one, two, three or four day R variants, each reflecting an infectious bite (for simplicity, we assumed each infectious

bite transmitted a single variant). We simulated the effect of MOI similarly, assigning each patient one through four day 0 variants and the same number of day R variants.

2. Monte Carlo uncertainty analysis

To accurately measure treatment success, estimates of the cure rate need to be adjusted for two types of misclassification: false positives (reinfections incorrectly being classified as recrudescent) and false negatives (true recrudescent infections misclassified as reinfections because a minority variant in the day 0 sample was not detected by nPCR). To adjust for this misclassification, we developed an uncertainty analysis that requires two sources of external, or prior, information: the distributions of false positives and false negatives. These distributions can be estimated using data from antimalarial efficacy studies.

We developed a method for estimating the distribution of false positives that reflects our understanding of the factors that influence the probability of a chance match and exploits characteristics of the study data themselves, allowing the probability of a false positive to appropriately be tailored to the study setting. False positive probabilities were calculated using the same simulation procedure described above, except that the number of allelic variants observed in each patient at day 0 and day R, and the population-wide distribution of allelic variants were set to match study data. We used MATLAB R2008a (Natick, MA) to simulate the infection and reinfection of N patients, where N was the number of patients who participated in the study. Each patient was assigned X day 0 and Y day R infections from the observed

day 0 and day R distributions of allelic variants (the X for each patient was randomly selected from the observed distribution of the number of day 0 infections, the Y randomly selected from the distribution of the number of day R infections) and tested for matches. The false positive probability for this simulated study was then calculated as the number of chance matches divided by N. We repeated this process 100,00 times (generating 10,000 false positive probabilities) to create the distribution of the proportion of recrudescent infections that were false positives.

To estimate the distribution of false negatives, we made use of the observation that nPCR has limited sensitivity to variants comprising less than 20% of a patient's parasite population.(30, 31) Misclassification of a recrudescence as a reinfection, a false negative, requires that each day R variant be undetected in the day 0 variants, as a single shared variant will result in the classification of the recurrence as a recrudescence according to the MMV/WHO guidelines.(20) To our knowledge, the only published information on the role of false negatives comes from Juliano *et al.* who used heteroduplex tracking assays (HTAs), a molecular method more sensitive to minority variants and genetic variation than nPCR, and found that five of six new infections (83%) identified by PCR-correction were truly recrudescent infections.(29) However, their study population was at negligible risk of reinfection, likely making their results an overestimate in the context of an average antimalarial trial. Therefore, to estimate the proportion of reinfections that were false negatives we used the median number of variants observed in the day R samples, assumed each variant carried with it a 20% chance of being missed in the day 0 sample, and calculated the probability that all were missed at day 0 resulting in a false negative using this

formula: proportion of false negatives equals $(0.2)^v$, where v is the median number of variants. The 20% chance was based on existing literature and expert opinion. Figure 5 shows our estimate of the effect of the number of variants in the day R sample on the probability that a recrudescence was misclassified as a reinfection (a false negative). We also conducted a sensitivity analysis varying the probability of a band being missed in the day 0 sample from 0 to 80%.

We conducted a Monte Carlo uncertainty analysis to adjust the observed number of recrudescent infections as determined by PCR-correction after genotyping *msp2* by the estimated distributions of false positives and false negatives. Using an approach similar to that described by Jurek *et al*.,(36) we calculate the adjusted cure rate using this formula:

$$\text{Adjusted cure rate} = [N_t - (N_{recru} - (N_{recru} \times FP) + (N_{new} \times FN))] / N_t \quad (1)$$

Where N_t is the total number of patients, N_{recru} is the number of recrudescent infections identified by PCR-correction, FP is the proportion of recrudescent infections that were false positives, N_{new} is the number of reinfections identified by PCR-correction, and FN is the proportion of reinfections that were false negatives.

We used Oracle Crystal Ball, Fusion Edition (Redwood Shores, CA) software to run 100,000 trials in which the number of recrudescent infections as determined by PCR-correction after genotyping *msp2* in each study area was adjusted and the cure rate calculated using formula 1 (above). As the last step in each trial, we included a bootstrap step to allow for sampling error by generating a random value from a

binomial distribution in which the number of trials was equal to the number of patients in the study and the probability of success was the uncertainty-adjusted probability of treatment failure. This approach is appropriate for estimating the sampling error of an estimated proportion.(74) These random values were then used as the number of recrudescences to calculate the final cure rate, adjusted for both uncertainty and sampling error. We also ran 100,000 trials without the bootstrap step to explore the effect of uncertainty in the absence of sampling error, and finally, also calculated traditional 95% confidence intervals around the PCR-corrected cure rate with no adjustment for outcome misclassification to demonstrate the effect of sampling error in the absence of uncertainty about the outcome.

3. Example data

To provide an example of our proposed uncertainty analysis, we used genotyping data from two randomized antimalarial efficacy trials conducted in areas of differing transmission intensity. The data from the high transmission area came from a study in Tororo, Uganda (N=401); the researchers were comparing the efficacy of an amodiaquine plus artesunate regimen compared to an atemether-lumefantrine regimen.(4) The data from the low transmission area were generated by a study conducted in Bobo-Dioulasso, Burkina Faso (N=827); the researchers were comparing the efficacy of amodiaquine, sulfadoxine-pyrimethamine and amodiaquine plus sulfadoxine-pyrimethamine.(75) In both studies, the different therapies did demonstrate different levels of efficacy(4, 75); however, because we are not interested in a particular treatment's efficacy, and instead are simply providing an

example of the uncertainty analysis, we did not stratify by treatment arm. The data for each patient included the number and identity of allelic variants. Greenhouse *et al*. used two sets of primers for amplification to capture two allelic families of *msp2*, IC3D7 and FC27.(5) Alleles were considered different if they were from different allelic families or if they were not the same length.

D. Results

1. Characteristics affecting the probability of false positives

The simulations compared the effect of transmission intensity and MOI on the probability of a false positive across ten negative binomial distributions comprising alleles with the same mean size (350bp) but different variances (Figure 4); increased variance signified higher levels of allelic (genetic) diversity in the population under study. We drew 100,000 samples of allelic variants from each distribution, assumed 100% treatment success, and drew a second variant to allow us to calculate the probability of a false positive. We calculated these probabilities at different levels of transmission intensity and different MOI.

At any level of allelic variance, the greater the number of post-treatment bites, or the more variants a patient had at day 0 and day R, the more likely a false positive. Conversely, higher levels of allelic diversity had lower probabilities of false positives regardless of transmission intensity or MOI (Figure 6).

2. Example of Monte Carlo uncertainty analysis

We used two data sets to provide examples of our Monte Carlo uncertainty analysis, which adjusted the number of recrudescent infections identified by PCR-correction by false positives (the proportion of nPCR identified recrudescent infections misclassified due to a variant in the day 0 and day R sample matching by chance) and false negatives (the proportion of nPCR reinfections misclassified due to nPCR insensitivity).

Patients from Tororo, the high transmission area, had one to eight day 0 variants (median of four) and one to eight day R variants (median of three). There were 40 variants in the day 0 sample when divided into 20 base pair bins with variants ranging in size from 181 to 1611bp (we had added 1000bp to variants amplified by primers specific to the FC27 allelic family to differentiate them from variants amplified by primers specific to the IC3D7 allelic family). There were 38 variants in the day R sample with sizes ranging from 212 to 1663bp.

Patients from Bobo-Dioulasso, the low transmission area, had one to eight day 0 variants (median of two) and one to six day R variants (median of two). There were 39 variants in the day 0 sample with sizes ranging from 195 to 1637bp. There were 25 variants in the day R sample with sizes ranging from 232 to 1565bp.

a. False positives

There was slightly less allelic diversity in Bobo-Dioulasso, however individuals with single pre-treatment and post-treatment variants had very similar probabilities of a false positive (in Tororo the probability was 0.050 vs. 0.045 in Bobo-Dioulasso). In patients with the sites' median numbers of pre-treatment and post-treatment variants (four and three respectively in Tororo; two and two in Bobo-

Dioulasso), the probability of a false positive was considerably higher in Tororo (0.327) when compared to Bobo-Dioulasso (0.163).

We used the probability of a day 0 and day R variant matching by chance to inform our distribution of false positives. We did this by running 10,000 simulations, each with the number of participants in the study. Each participant was assigned X day 0 and Y day R variants from the observed day 0 and day R distributions of allelic variants (the X for each patient was randomly selected from the observed distribution of the number of day 0 variants, the Y randomly selected from the distribution of the number of day R variants) and tested for matches. We created a distribution of these 10000 probabilities and determined its mean and standard error. The mean proportion of recrudescent infections that were false positives was 0.423 in Tororo (standard error = 0.0007) and 0.193 in Bobo-Dioulasso (standard error = 0.0004).

b. False negatives

False negatives occur when a minority variant is undetected by the nPCR and results in misclassification of a recrudescent infection as a reinfection. The proportion of variants likely to be false negatives was equal to $(0.2)^v$, where 0.2 is the probability that a variant was missed in day 0 and v is the median number of variants in the site's day R samples (Figure 5). The proportion of reinfections that were false negatives was 0.008 in Tororo and 0.04 in Bobo-Dioulasso.

c. Adjusted number of recrudescent infections

There were 232 recurrent parasitemias among the 401 study participants from Tororo. After genotyping *msp2,* 145 were classified as recrudescent and 87 as reinfection, corresponding to a cure rate of 63.8%. After conducting our uncertainty

analysis, we determined that the 95% simulation interval of likely cure rates ranged from 74.6 to 83.3% (Table 1).

Among the 827 study participants from Bobo-Dioulasso, there were 75 recurrent parasitemias. After genotyping *msp2*, 50 were classified as recrudescent and 25 as reinfection, corresponding to a cure rate of 94.0%. After conducting our uncertainty analysis, we determined that the 95% simulation interval of likely cure rates ranged from 93.5 to 96.5% (Table 1).

We evaluated the effect of uncertainty due to outcome misclassification and sampling error independently. The adjustment for uncertainty regarding outcome misclassification was responsible for the upward shift of the cure rate (indicating greater efficacy) and sampling error increased the width of the simulation interval (Table 1).

E. Discussion

Our simulations demonstrated the effect of allelic diversity, transmission intensity and MOI on the probability of a chance match between a day 0 and day R variant. False positives were more common in areas with less diverse parasite populations and high transmission levels which would lead to underestimation of cure rates in those areas. The most dramatic increase in the probability of a false positive was associated with increased MOI (Figure 6B).

The results of the proposed uncertainty analysis indicated false positives (reinfections misclassified as recrudescences) were responsible for the majority of misclassification in both examples. Selecting variants at random from the observed

distributions in Tororo resulted in false positives in more than one-third of the recrudescent infections, while in Bobo-Dioulasso the probability that a recrudescence was a false positive was less than 20%. The discrepancy is primarily the result of the lower median day 0 and day R MOI in Bobo-Dioulasso, as both areas had similar levels of allelic diversity. In Tororo, false positives resulted in an uncertainty interval of the cure rate that indicated greater efficacy than the original point estimate calculated after genotyping *msp2*.

False negatives (recrudescences misclassified as reinfections) resulted in only a small amount of misclassification for two reasons. First, multiple variants in the day R sample (observed in both study sites) decreased the probability of this type of misclassification exponentially (Figure 5). Our sensitivity analysis indicated that even with a 30% chance that a day 0 variant was not detected, the impact of the observed number of variants in the day R samples resulted in a negligible effect of false negatives (data not shown). As the chance a day 0 variant was not detected increased past 40%, the impact began to increase more rapidly, however values greater or equal to 30% are highly unlikely. Second, using PCR-correction there were very few recurrences identified as reinfections; regardless of the probability that a reinfection was truly a recrudescence, the contribution of this type of misclassification to overall uncertainty would be low. However, in areas of low transmission, such as Southeast Asia, where few variants are present a day 0, false negatives may be an important source of misclassification.(29)

The uncertainty analysis was based on PCR-correction of a single marker. Though the use of multiple markers to perform PCR-correction (a common practice)

may reduce the probability of false positives, it increases the probability of false negatives because the MMV/WHO guidelines state that a single marker classified as a reinfection results in the recurrence being classified as such, regardless of the classification of other markers genotyped. As additional information is generated regarding the probability of false negatives and how it changes with the use of multiple markers, it will be possible to refine this uncertainty analysis to accommodate multiple markers.

The impact of misclassification with regard to WHO efficacy thresholds varied between the two sites. Although ultimately the range of likely cure rates in Tororo did not cross a WHO cut-point, it did demonstrate that misclassification plays an important role. In Bobo-Dioulasso, the area of low transmission, a WHO cut-point was included in the interval of likely cure rates (i.e. 93.5 to 96.5%). The relatively few patients who had recurrent parasitemia in Bobo-Dioulasso resulted in a narrow interval of cure rates with values similar to the PCR-corrected point estimate, however a drug whose cure rate calculated the traditional way would have been just below the level of efficacy required for new drugs and may in turn have been rejected, when it should be eligible for consideration. Misclassification should always be considered when policy decisions are made based on estimates of efficacy.

Our approach to generating the distribution of false positives is probably not practical for use in all antimalarial efficacy studies. However, we are optimistic that it is possible to generate three reasonable "stock" distributions of false positives, one for high, medium, and low transmission areas. The uncertainty analysis itself is quite straightforward and can easily be carried out in Crystal Ball, a relatively inexpensive

addition to Microsoft Excel, and perhaps eventually in a free web-based tool. It is our hope that future molecular research will allow us to provide researchers with distributions of false positives and false negatives, making this uncertainty analysis available for wide use.

Misclassification of recurrent parasitemias resulting from PCR-correction has been previously described. Adjustments of PCR-corrected trial results have been made using the distribution of allelic variants to calculate the probability of false positives leading to incorrect classification of the recurrence as a recrudescence when it is a reinfection.(5, 21, 28, 33) HTAs, which use radiolabeled probes to bind to host amplicons, are more sensitive to minority variants and genetic variation than nPCR(21, 30, 86, 87) and have been used to demonstrate that nPCR insensitivity can result in recrudescent infections being misclassified as reinfections.(29) To our knowledge, simultaneous adjustment for both types of misclassification has not been undertaken previously.

Traditional confidence intervals summarize only the effect of random error and do not capture or reveal any uncertainty resulting from bias, including misclassification or measurement error, in the study. Adjusting results for misclassification was illustrated in previous work(36) and is grounded in methods proposed to estimate intervals that are an extension of traditional confidence intervals through use of simulations.(34) Some researchers are uncomfortable with the explicit assumptions about misclassification that are required for uncertainty analyses. However, this approach is far preferable to assuming misclassification is entirely

absent, an implicit assumption in the traditional estimation of a PCR-corrected cure rate.

A point estimate of the cure rate, the traditional outcome measure in antimalarial efficacy studies, is insufficient given the limitations of PCR-correction. This insufficiency is even more important given the policy implications of efficacy estimates. A 95% simulation interval for the cure rate, instead of an estimate likely to be biased by outcome misclassification, may encourage more careful assessment of a treatment's utility before policy decisions are made. This work provides a template for adjusting for outcome misclassification in antimalarial efficacy studies that addresses both types of misclassification and can be applied to any study data that include information on the variants present in the patient population.

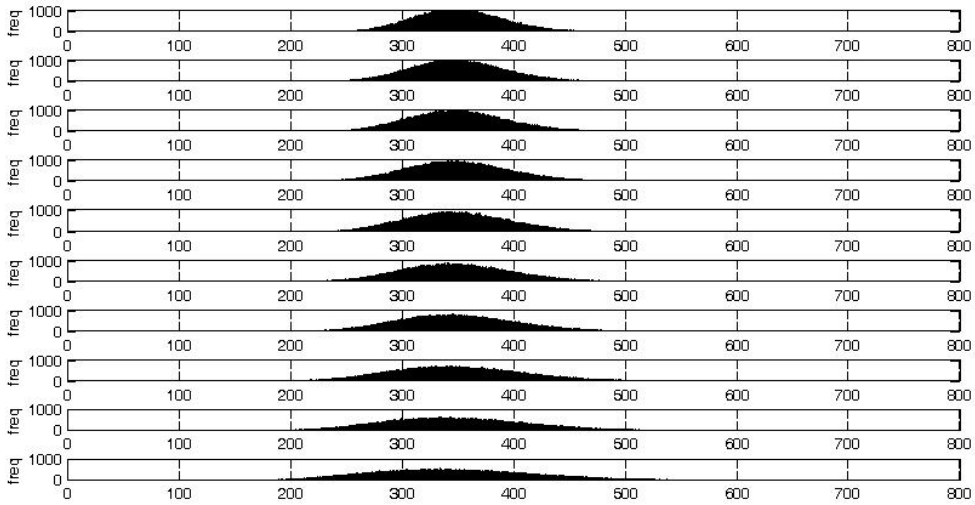

Figure 4. The ten negative binomial distributions of base pairs used for simulations. These distributions all have the same mean (350 bp); the variance increases from top to bottom. Each plot represents 100000 randomly assigned number of base pairs selected from the distribution. x-axis: number of base pairs; y-axis: frequency

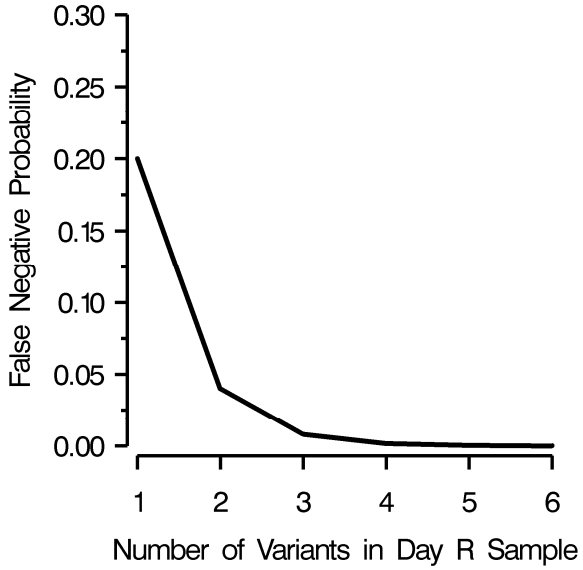

Figure 5. Multiplicity of infection and its effect on the probability of a false negative. A false negative, or a recrudescent infection misclassified as a reinfection, occurs as a result of nPCR insensitivity to minority variants (those comprising less than 20% of a patient's infection).

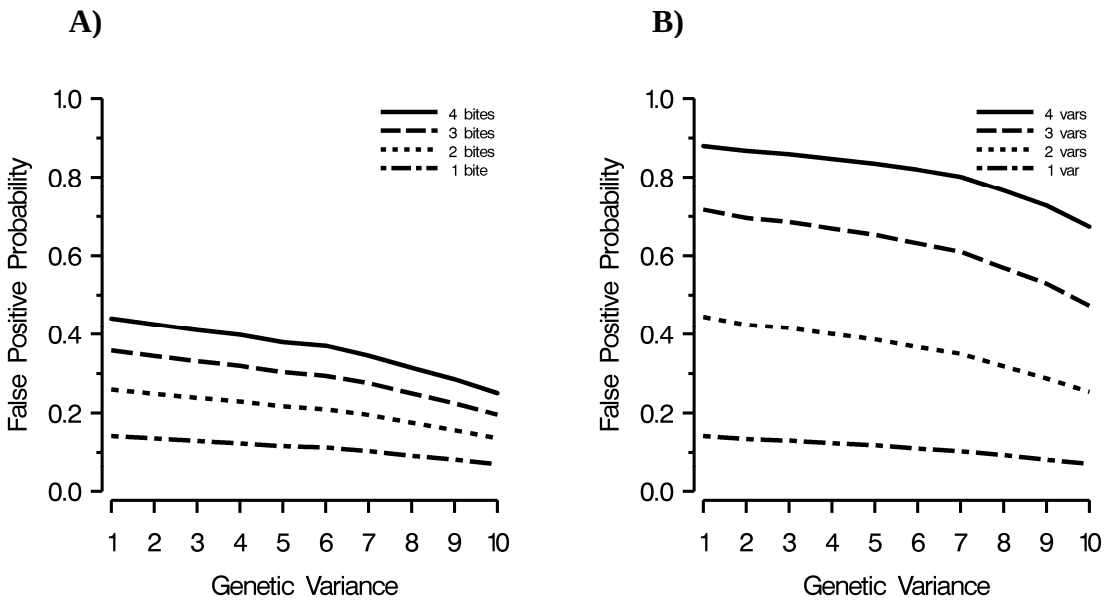

Figure 6. A) The effect of transmission intensity on the probability of a false positive, B) The effect of multiplicity of infection on the probability of a false positive. x-axis: measure of allelic variation in parasite population (1 = least variance); y-axis: probability of a false positive (a false positive occurs when a day 0 and day R variant match by chance); var = variant

Table 2. Results from the uncertainty analysis: estimates of cure rates from studies in Bobo-Dioulasso, Burkina Faso, and Tororo, Uganda.

	Tororo N = 401	**Bobo-Dioulasso N = 827**
Number of recurrent infections	232	75
Number of recrudescent infections identified by PCR-correction based on genotyping of *msp2*	145	50
Cure rate (95% CI[a]) calculated by PCR-correction based on genotyping of *msp2* (%)	63.8 (59.0 – 68.4)	94.0 (92.1 – 95.4)
95% SI[b] of likely cure rates adjusted for only for uncertainty (%)[c]	77.4 – 80.5	94.9 – 95.1
95% SI of likely cure rates generated by Monte Carlo uncertainty analysis (%)	**74.6 – 83.3**	**93.5 – 96.5**

a. CI = confidence interval; b. SI = simulation interval; c. This interval does not take sampling error into account.

CHAPTER 4

HIV-1 PROTEASE INHIBITORS AND INCIDENT MALARIA: AN ANCILLARY STUDY TO ACTG5208

A. Summary

HIV-1 protease inhibitors (HIV PIs) have antimalarial action *in vitro* and in murine models. The effect of HIV PIs on malaria has never been studied in humans.

We used data from ACTG5208 to compare incidence of clinical malaria in HIV-infected adult women randomized to PI-based antiretroviral therapy (ART) to women randomized to non-nucleoside reverse transcriptase inhibitor (NNRTI)-based ART. We used pooled logistic regression to calculate hazard ratios and 95% confidence intervals. We conducted a stratified analysis and explored effect measure modification by seasonality and concomitant medication use.

ART assignment was not associated with the hazard of malaria (HR = 1.03; 95% CI: 0.73 - 1.44). This finding was similar in analyses stratified by trial [Trial 1, HR = 1.37 (95% CI = 0.76 - 2.44); Trial 2, HR = 0.94 (95% CI = 0.62-1.43)]. There was no modification of the HR by seasonality or use of concomitant medications.

Clinical malaria among HIV-infected individuals was not reduced by PI-based ART. This study is the first step in understanding whether HIV PIs will reduce

malaria and additional work focused on incidence of sub-clinical malaria and malaria in children may be warranted.

B. Introduction

HIV and malaria are highly co-prevalent in some regions of the world, including Sub-Saharan Africa. These infectious agents interact biologically such that each may increase incidence of the other.(42) A treatment efficacious against both diseases or an antiretroviral that provided prophylaxis for malaria would offer a tremendous advantage to the millions of HIV-infected people in areas where co-infection is common.

The biological interaction between the two diseases is well documented though incompletely understood. HIV infection increases incidence and worsens clinical manifestations of malaria(43-45); malaria elevates HIV viral load(47, 48) and decreases CD4 counts.(51) The data on drug interactions between antiretroviral therapies (ART) and antimalarials are incomplete. However, there are examples of harmful effects for the patient and alterations in the pharmacokinetics of the treatments, reviewed in (54) The biological and pharmacological interactions make a treatment effective against both diseases highly desirable. There is evidence that HIV protease inhibitors (PIs) may fill that role.(58, 59, 61-63, 71)

Evidence that aspartic PIs (the group of PIs used in HIV ART) have antimalarial effects on *Plasmodium falciparum*, the most virulent of the human malaria-causing parasites, was first generated more than two decades ago.(88) More recent *in vitro* studies have demonstrated that HIV PIs inhibit the growth of *P.*

falciparum, regardless of the parasites' sensitivity to antimalarials(58, 59), and parasites exposed to sera taken from HIV-infected patients being treated with LPV/r had a 50 to 95% reduction in growth.(62) *In vivo* evidence from murine models also supports the antimalarial effect of HIV PIs.(61, 63) Nathoo *et al.* proposed that HIV PIs may have an impact on patients with malaria independent of antiparasitic effects after observing *in vitro* that treating cells with HIV PIs resulted in a marked reduction in the expression of CD36, a human cell receptor associated with the binding of malaria parasites.(72)

Protease inhibitors are not currently recommended for first-line ART use in resource limited settings.(38) However the recent advent of heat stable LPV/r, coupled with observed resistance to first-line non-nucleoside reverse transcriptase inhibitor (NNRTI)-based ART (39) and resistance to nevirapine (NVP) associated with use of single dose NVP for prevention of mother-to-child transmission of HIV(89)**,** makes it likely the use of PIs in the developing world will increase. If the antimalarial effect of HIV PIs is also found to be present in humans, their use in regions with endemic malaria would be even more valuable.

The Adult AIDS Clinical Trials Group (AACTG) recently completed two phase III randomized clinical trials (ACTG5208) to compare the antiretroviral activity of LPV/r-based ART to NNRTI-based ART in HIV-infected women who either had been exposed to single dose NVP (Trial 1) or were treatment-naïve (Trial 2). We conducted an ancillary study using the AACTG data to measure the association between LPV/r and clinical malaria in humans

C. Methods

1. ACTG5208.

The study consisted of two trials that followed participants for 48 weeks after the final participant was randomized.(40) Trial 1 enrolled women who had received single-dose NVP to prevent mother-to-child transmission of HIV (N=243); Trial 2 enrolled women with no history of NVP exposure (N=500).(40) In both trials, women were randomized 1:1 to receive either LPV/r-based ART or NNRTI-based ART. All participants received tenofovir DF and emtricitabine consistent with World Health Organization (WHO) guidelines.(52) Participants had regularly scheduled study visits (at weeks 2, 4, 8, 12, 16, 24 and then every 12 weeks) and could also report to the study site for medical care as needed.

2. Study population.

ACTG5208 enrolled women greater than 13 years of age (or older as dictated by the study site IRB) with a CD4+ cell count < 200 cells/mm^3 obtained within 90 days prior to study entry. Enrollment criteria are described in detail elsewhere.(40) We used data from participants in both trials from all of the study sites where malaria is endemic: Eldoret and Kericho, Kenya; Lilongwe, Malawi; Kampala, Uganda; Lusaka, Zambia; and Harare, Zimbabwe.

3. Exposure and outcome.

The main exposure was the therapeutic regimen to which the participant was randomized (i.e., LPV/r-based ART or NNRTI-based ART). The primary outcome

was clinical malaria. The trial staff classified this diagnosis as probable or confirmed. Probable malaria required both a "compatible clinical syndrome" and use or recommendation of antimalarial treatment.(40) Confirmed malaria required both "compatible clinical syndrome" and identification of *Plasmodium* sp. on a peripheral blood smear.(40) We also included patients who were prescribed antimalarials (amodiaquine, artemether/lumefantrine, artesunate, chloroquine phosphate, dihydroartemisinin/piperaquine, mefloquine HCl, pyrimethamine/sulfadoxine, quinine dihydrochloride, quinine sulfate or sulphalene/pyrimethamine) without a recorded diagnosis of malaria as probable cases.

4. Statistical analysis.

We counted person-time at risk from treatment initiation until the date of malaria, death, drop-out, or study completion. We used the hazard ratio (HR) as a measure of association and the 95% confidence interval (CI) as a measure of precision. To obtain the hazard ratio we fit pooled logistic regression models, which approximate Cox proportional hazards models(90) as long as the event proportion in all discrete time periods is less than 10%.; in our study the largest event proportion was 5.6%. Time-on-treatment was modeled using a 5-knot restricted cubic spline (Harrel's DASPLINE(84)) to allow a flexible nonlinear association between time and malaria and all models included trial and study site. Because few patients had greater than 165 weeks of follow-up and there were no incidences of malaria after that time, we administratively censored all participants still at risk at 165 weeks.

We examined seasonality and concomitant medication use as possible effect measure modifiers by the addition of product terms with exposure. Using climate data from the National Oceanic and Atmospheric Administration and evidence from the literature(78-82), we created a time-varying dichotomous variable denoting rainy season (indicating a higher risk of malaria transmission). We also created a time-varying dichotomous variable indicating current use of concomitant medications with antimalarial activity (azithromycin, clindamycin, diaminodiphenylsulfone, doxycycline hydrochloride, doxycycline monohydrate or trimethoprim/sulfamethoxazole). We examined the proportional hazards assumption by a plot of the log cumulative hazard by time as well as the addition of terms for the products of exposure and time (including spline coefficients). We conducted a stratified analysis in which we explored the effect of LPV/r in Trial 1 and Trial 2 separately. As a sensitivity analysis, we fit a model in which only confirmed malaria cases were considered to have experienced a malaria episode. Finally, we generated plots of survival by time for each exposure group. All analyses were conducted using SAS statistical software (version 9.2; SAS Institute, Cary, NC).

D. Results

There were 145 women enrolled in Trial 1 from the relevant sites. They were split almost evenly between treatment arms, were similarly aged, and had comparable baseline CD4 counts and HIV viral loads (Table 3). Participants in Trial 1 were followed for up to 144 weeks; the average duration of follow-up was

63 weeks. In Trial 1, 48 participants (33%) were identified as having probable or confirmed malaria at least once during follow-up (Table 4). Of those, 20 (42%) participants had more than one episode of malaria.

There were 301 women enrolled in Trial 2 from the relevant sites (Table 3). There were seven more women randomized into the LPV/r-based ART arm compared to the NNRTI-based ART arm. The average age in Trial 2 was slightly higher than in Trial 1 but similar across treatment arms within Trial 2. Baseline CD4 counts and viral loads were similar although there was a slightly higher proportion of patients with a baseline CD4 count < 50 cell/mm^3 in the LPV/r arm (Table 1). Participants in Trial 2 were followed for up to 185 weeks; 23 were administratively censored at 165 weeks. The average duration of follow-up was 88 weeks. In Trial 2, 89 participants (30%) were identified as having probable or confirmed malaria at least once during follow-up (Table 4). Of those, 41 (46%) participants had more than one episode of malaria.

The proportional hazards assumption was met. Neither seasonality nor concomitant use of medications with antimalarial effect modified the effect of treatment assignment on the hazard of malaria (results not shown). When we adjusted for trial and site, treatment assignment was not associated with the hazard of malaria (HR = 1.03; 95% CI: 0.73 - 1.44). A survival curve in which trial and site are collapsed is presented in Figure 7. Trial and site-specific curves are available in Appendix H.

When we considered only laboratory confirmed cases of malaria, the results were very similar; there was no effect of treatment assignment on the

hazard of malaria (HR = 1.28; 95% CI: 0.58 - 2.82). This finding was similar in analyses stratified by trial [Trial 1, HR = 1.37 (95% CI = 0.76 - 2.44); Trial 2, HR = 0.94 (95% CI = 0.62-1.43)]. Trial-specific curves are presented in Figure 8.

E. Discussion

Laboratory evidence supports the antimalarial effect of HIV PIs. The effect has been shown in cultured parasites, both drug sensitive and drug resistant, and in mice using two murine species of *Plasmodium*. Skinner-Adams *et al*. reported that ritonavir (RTV), one of the PIs used in ACTG5208, and another HIV PI inhibited growth in parasites *in vitro* whereas the antiretroviral NVP had no effect.(58) These findings have been replicated and additional HIV PIs have demonstrated antimalarial effects at clinically relevant concentrations.(59, 62)

Studies of murine models of malaria also have demonstrated the antimalarial effect of HIV PIs. After infection with *P. chabaudi*, mice exposed to LPV/r had delayed onset of parasitemia by two days and a decrease in median parasitemia from 20% to 4%.(61) Evidence from a different murine model, *P. yoelii*, suggested that HIV PIs, including LVP, the other ACTG5208 PI, inhibit growth of preerythrocytic-stage parasites.(63)

Unlike these laboratory studies, we saw no evidence of an antimalarial effect. Patients randomized to LPV/r-based ART were as likely to develop malaria as those randomized to receive NNRTI-based ART.

We propose two potential reasons that may help to explain why no effect was observed. One possibility is that any antiparasitic action of the LPV/r occurs simultaneously with a reduction in the innate immune response to malaria resulting

from exposure to LPV/r, resulting in a null net benefit. Nathoo *et al.* proposed that although the decrease in CD36 expression may be beneficial to the patient because of decreased cytoadherence of parasitized erythrocytes, it could also make it more difficult for a patient's innate immune system to fight *P. falciparum* infections because of reduced phagocytosis which also resulted from the diminished expression of CD36.(72)

Another possibility is that the antimalarial effects of PIs are highly concentration-dependent. The *in vitro* studies of HIV PIs on malaria parasites strove to explore the effect using clinically relevant concentrations, including trough concentrations.(58, 59, 61, 62) However it is unknown if the parasites' exposures to drugs in the laboratory studies are truly equivalent to the fluctuating serum drug concentrations one would anticipate in a patient on ART. Carefully controlled laboratory experiments, isolated from the complex biological interactions among drugs, host acquired and innate immunity, and *in vivo* parasite growth dynamics, may simply not approximate the experience of HIV-infected patients on PI-based ART.

Women assigned to NNRTI-based ART in Trial 1 were more likely to experience virologic failure when compared to the women randomized to LPV/r-based ART.(CROI 2009 abstract) This reduced control of HIV disease could have an impact on our findings as increased incidence of clinical malaria has been associated with low CD4 counts in HIV-infected individuals.(43, 45) Had we observed a protective effect of LPV/r, it would have been important to consider that it may simply have resulted from the superior virologic response to that therapy. It is of note that in Trial 2, LPV/r-based ART and NNRTI-based ART had comparable virologic

efficacy (CROI 2010 abstract) and in our stratified analyses there was no effect of LPV/r on incidence of malaria in either trial.

Women who experienced virologic failure, as well as those with adverse reactions to their assigned medications, were eligible to switch antiretroviral therapies and receive the treatment available in the other study arm. Among the 346 women included in this ancillary analysis, 50 (14%) did so; almost all moved from NNRTI-based ART to LPV/r-based ART. Because it is a relatively small percentage of patients who switched treatments and because fewer than 10% of the malaria episodes occurred after the participant had switched, we do not anticipate that this had a considerable effect on our findings.

This work has several limitations primarily related to the outcome. The sensitivity and specificity of clinical syndrome when used to diagnose malaria are notoriously limited (Steve Taylor, personal communication, (91)). When an exposure is dichotomous, nondifferential misclassification (we do not anticipate the sensitivity and specificity would vary across treatment arm) usually drives effect estimates towards the null. It is possible that this partially explains our null results. Additionally, the ACTG5208 study staff did not regularly collect blood smears as part of the protocol; instead they collected them when testing for malaria was appropriate based on the site's standard of care guidelines. The lack of systematically collected blood smears and the absence of information with regard to blood smears that were negative, made slide-positivity an unacceptable primary outcome.

This work is only the first step in understanding the antimalarial effects of HIV PIs in humans. "Probable" malaria without laboratory confirmation is an

imperfect outcome. However, the comparable effect estimates generated by models that included both probable and confirmed cases, and the sensitivity analysis in which only confirmed cases were considered to have malaria strengthen our findings.

The use of biological markers will allow future investigations into the impact of HIV PIs on sub-clinical malaria. Further research is also warranted on the effects of these drugs in children, who are at greater risk of clinical malaria. Additionally, laboratory evidence suggests that co-administering HIV PIs with chloroquine or mefloquine may enhance the antimalarial activity of the drugs even in resistant parasites; perhaps the utility of HIV PIs as antimalarials will result from co-administration with existing therapeutics. The optimism about HIV PIs having an antimalarial effect in HIV-infected individuals may need to be tempered, but there is still much to be learned.

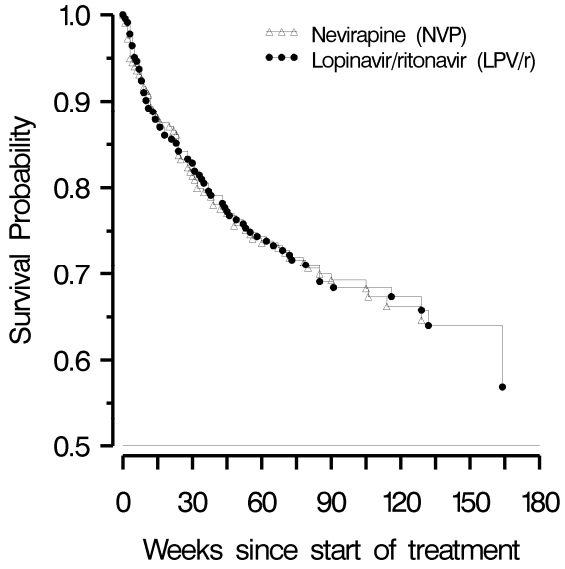

Figure 7. Survival curves by treatment assignment for participants in ACTG5208.

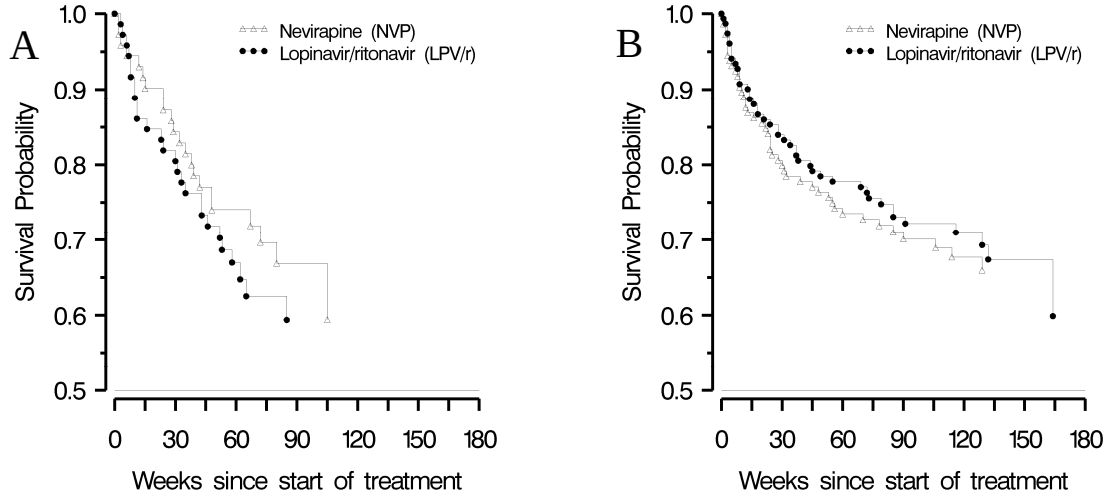

Figure 8. Trial-specific survival curves for participants in ACTG5208. (A) Trial 1. (B) Trial 2.

Table 3. Participants in ACTG5208 by trial and treatment group from sites with endemic malaria.

	Trial 1 (n = 145)		Trial 2 (n = 301)	
	LPV/r[a] (n = 72)	NPV[b] (n = 73)	LPV/r[a] (n = 154)	NPV[b] (n = 147)
Age, years	30.5	30.7	35.8	34.8
Site, n				
Eldoret, Kenya	9	8	25	22
Kericho, Kenya	14	13	23	23
Lilongwe, Malawi	10	14	22	22
Kampala, Uganda	9	8	21	22
Lusaka, Zambia	12	12	21	19
Harare, Zimbabwe	18	18	42	39
Baseline CD4, mean cells/mm^3 ± SD[c]	126 ± 57	134 ± 62	123 ± 78	127 ± 81
Baseline CD4 <50, n(%)	7 (9.7)	10 (13.7)	27 (17.5)	20 (13.6)
Baseline HIV-1 RNA, median copies/mL[d]	157,453	161,630	131,175	112,401

[a]Lopinavir boosted with ritonavir–based antiretroviral therapy.

[b]Nevirapine–based antiretroviral therapy.

[c]CD4-positive cells per mm^3. [d]Log HIV-1 viral load.

Table 4. Clinical malaria among participants of ACTG5208.

	Trial 1 n = 145		Trial 2 n = 301	
	LPV/r (n)	NVP (n)	LPV/r (n)	NVP (n)
All malaria, n				
Eldoret, Kenya	5	5	5	8
Kericho, Kenya	8	6	12	10
Lilongwe, Malawi	7	5	7	8
Kampala, Uganda	3	0	6	9
Lusaka, Zambia	3	6	12	8
Harare, Zimbabwe	0	0	2	2
Total	26	22	44	45
Confirmed malaria, n	4	3	10	8

CHAPTER 5

DISCUSSION

A. Factors influencing PCR-corrected cure rates in antimalarial efficacy trials

1. Summary of findings

We identified factors that influence outcome misclassification and used them to develop a Monte Carlo uncertainty analysis; our findings suggested that false positives (incorrectly identified treatment failures) are common in antimalarial efficacy studies and result in underestimates of treatment efficacy. We conducted an intuition-building exercise which relied exclusively on simulated data. As anticipated, holding transmission intensity and multiplicity of infection constant, increased allelic diversity reduced the probability of the same variants being observed before and after treatment by chance (a false positive). Conversely, holding allelic diversity constant, increased transmission intensity and multiplicity of infection increased that probability. From our simulations, it was clear that we would need to consider these factors in the development of our Monte Carlo uncertainty analysis.

During the development of the Monte Carlo uncertainty analysis, we relied heavily on statistical methods employed by Jurek *et al*.(36) and probability-based corrections of molecular data used by malariologists(5, 21, 28, 33). Having identified important factors associated with outcome misclassification in our simulation exercises and used the literature to estimate the likely role of PCR-insensitivity to

minority variants, we worked to design a practical approach that could be useful to clinicians and others associated with antimalarial treatment trials.

The model we created used a straight-forward approach, with some technical details: (1) remove the proportion of infections believed to be false positives from the pool of study participants classified as treatment failures by PCR (this requires a distribution of the probability of false positives); (2) move the proportion of infections believed to be false negatives from the pool of patients classified as reinfections into the pool of treatment failures (this requires an estimate of the probability of false negatives); (3) use the adjusted pool of treatment failures to calculate a new cure rate; (4) use a nonparametric bootstrap step to generate random error around the cure rate; (5) repeat this process 100,000 times and generate a 95% simulation interval of likely cure rates.

When we conducted the uncertainty analysis using data from two separate trials conducted in low and high transmission areas, we discovered that the probability of false positives was quite high in the high transmission site (33%) and lower but still unexpectedly high in the low transmission site (16%). In both sites, the likely impact of false negatives appeared negligible. The initial estimate of the cure rate in the high transmission area was 63.8%; after we used our uncertainty analysis to adjust the estimate for outcome misclassification, the 95% simulation interval of the cure rate was 74.6 to 83.3%. The initial estimate of the cure rate in the low transmission area was 94.0%; after the uncertainty adjustment the 95% simulation interval of the cure rate was 93.5 to 96.5%.

2. Findings in the context of current literature

It is not uncommon in the literature to find mention of the possibility of outcome misclassification, especially the erroneous classification of new infections as treatment failures, but it is almost always dismissed as unimportant. Publications that used data on the distribution of allelic variants in the parasite population to adjust estimates of treatment efficacy beyond those generated by PCR-correction have shown that using this additional source of information to assess the probability of false positives likely improves estimates of the cure rate.(5, 21, 28, 33)

This project builds on those studies by incorporating not only information related to the probability of false positives but also uses data on PCR-insensitivity(30, 31) to estimate the impact of false negatives. To our knowledge, there is only one paper that has addressed the impact of false negatives and they estimated that a majority of "reinfections" were truly treatment failures.(29) This is quite different then our findings, in which only a small percentage (1 to 4%) of patients were likely misclassified in that way. However, their study population was not at risk for reinfection due to hospitalization throughout follow-up or very low local transmission intensity; this makes their results not generalizable to the majority of treatment trials.

3. Strengths and limitations

a. Strengths

The uncertainty analysis we developed is straight-forward and, if study site-appropriate distributions of false positives and negatives are provided, can be executed with little statistical expertise or knowledege of statistical software.

The ability to exploit characteristics of the study data themselves also makes this analysis a desirable option. This allows for the inclusion of information we know to be important to misclassification, namely the distribution of genetic variants and the MOI. Additionally, explicit assumptions regarding the degree of misclassification is unappealing to some researchers. By tailoring these assumptions to the study site, and incorporating data that have been shown to be relevant to outcome misclassification, we demonstrate that these estimates of misclassification are not solely the results of "stastistical hand-waving."

b. Limitations

Malaria biology is complex. Human host immunity, genetic diversity of parasites within a single infectious bite, and sequestration and synchronicity of parasites, are only a few of the factors at work in the complicated dynanmics of malaria infection. Our analysis cannot address many of those factors and requires a number of assumptions.

One particular component of the analysis which we believe could be improved upon as additional data are generated is the role of false negatives. There is no relevant evidence to demonstrate their impact on study populations at risk for reinfection. Using information on the insensitivity of nPCR to minority variants and relying on the WHO/MMV guidelines on classifying a recurrent infection as new, we were able to estimate what we hope is a reasonable approximation, but as additional molecular data become available that are generated using very sensitive and precise techniques to identify all variants present in a host it is likely an updated approximation would be closer to the truth.

Our objective was to develop a tool that would be useful for clinicians and clinical trial specialists and would not require a great deal of statistical sophistication. Once the distributions of false positives and negatives have been developed, the actual adjustments we made are uncomplicated. However, the generation of the distributions of false positives required a significant amount of programming in MATLAB R2008a (Natick, MA). The programming would not be difficult to anyone accustomed to statistical software but it may be a disincentive to those without the inclination.

4. Implications

This project demonstrated that false positives may indeed have a large effect on estimates of the cure rate and that as currently calculated, PCR-corrected cure rates may underestimate drug efficacy.

If reliable estimates of the distributions of false positives and negatives can be generated for a range of transmission areas, this project lays the groundwork for the development of a web-based tool to be used by antimalarial efficacy researchers. Members of our laboratory are in the process of developing highly sensitive molecular techniques to determine which variants are in a patient's sample and they can then compare those findings with the variants identified by PCR. These data will allow us to generate transmission intensity-specific probabilities of false negatives and we are also considering developing additional transmission intensity-specific distributions of false positives. We would then collaborate with programmers to generate a web-based tool that would allow researchers to enter their study results

based on PCR-correction and estimates of transmission intensity for their study site and have the uncertainty analysis run for them.

Whether use of this analysis is adopted will rely on whether trialists and other researchers believe that it makes sense to incorporate this type of correction for outcome misclassification.

If nothing else, this work demonstrates that ignoring outcome misclassification, as is so commonly done in this area of study, results in erroneous findings. This makes the approach of using stringent cutpoints based on PCR-corrected cure rates to determine what is a suitable antimalarial treatment policy undesirable.

B. The effect of HIV-1 protease inhibitors on incidence of malaria

1. Summary of findings.

There was no association between receipt of PI-based ART and incidence of malaria in this study. We used pooled logitic regression to conduct a survival analysis comparing the incidence of clinical malaria between HIV-infected women assigned to LPV/r-based ART and women assigned to NNRTI-based ART. We saw no evidence of an effect when we considered both confirmed and probable cases (HR = 1.03; 95% CI: 0.73 - 1.44), nor in our sensitivity analysis in which only confirmed cases were considered to have malaria (HR = 1.28; 95% CI: 0.58 - 2.82). In analyses stratified by trial, we also saw no effect [Trial 1, HR = 1.37 (95% CI = 0.76 - 2.44); Trial 2, HR = 0.94 (95% CI = 0.62-1.43)].

We assessed possible modification of the hazard ratio by seasonality and use of concomitant medications but did not observe such modification.

2. Findings in the context of current literature

This is the first time the effect of HIV PIs on incidence of malaria in humans has been studied. Our finding of no effect is inconsistent with laboratory results. In cultured parasites, both drug sensitive and drug resistant, and in mice using two murine species of *Plasmodium*, HIV PIs inhibit parasite growth.(58, 59, 61-63) Skinner-Adams *et al*. first reported that RTV, one of the PIs used in ACTG5208, as well as another HIV PI inhibited growth in parasites(58); these findings have been replicated and additional HIV PIs have demonstrated antimalarial effects at clinically relevant concentrations.(59, 62)

Animal studies have also demonstrated the antimalarial effect of HIV PIs. After infection with *P. chabaudi*, mice exposed to LPV/r had delayed onset of parasitemia by two days and a decrease in median parasitemia from 20% to 4%.(61) Evidence from a different murine model, *P. yoelii*, suggested that HIV PIs, including LPV, the other ACTG5208 PI, inhibit growth of preerythrocytic-stage parasites.(63)

There is, however, one article to our knowledge that may be consistent with our findings. Nathoo *et al.* proposed that although the decrease in CD36 expression associated with exposure to LPV/r *in vitro* may be beneficial to the patient because of decreased cytoadherence of parasitized erythrocytes, it could also make it more difficult for a patient's innate immune system to fight *P. falciparum* infections because of reduced phagocytosis which also resulted from the diminished expression.

(72) Perhaps antiparasitic action of the LPV/r occurs simultaneously with a reduction in the innate immune response to malaria, resulting in a null net benefit.

3. Strengths and limitations

a. Strengths

This was the first time the effect of HIV PIs on malaria in humans has been studied. We had the benefit of longitudenal data from two randomized controlled trials conducted by the Adult AIDS Clinical Trials Group with follow-up times exceeding two years. The use of intent-to-treat analysis allowed us to assess the impact of LPV/r therapy without confounding by other factors; randomized experiments are generally regarded as the gold standard for estimating a causal effect. Additionally, the drug used in the comparison group (NVP) has previously demonstrated no antimalarial action (Tina 2004 JID, Redmond AIDS 2007) easing interpretation of our results.

b. Limitations

Clinical malaria is not an ideal outcome. The sensitivity and specificity of clinical syndrome when used to diagnose malaria are notoriously limited **(91),Steve Taylor, personal communication)**. The ACTG5208 study staff did not regularly collect blood smears as part of the protocol; instead they collected them when testing for malaria was appropriate based on the site's standard of care guidelines. The lack of systematically collected blood smears and the absence of information with regard to negative blood smears, made slide-positivity an unacceptable primary outcome.

As a part of our future research plan, we hope to explore sub-clinical malaria in the cohort of patients we used in this ancillary analysis. This will require a serological definition of malaria. Initially, antigen testing (specifically testing for HRP2) was considered, though we now believe the serum samples that are available to us from the ACTG5208 will not be appropriate. Our laboratory collaborators are currently working on looking at antibody titers over time to identify acute malaria infections and using rapid diagnostic test kits; in the future PCR may also be considered. In addition to providing information on sub-clinical disease, these types of data likely have greater sensitivity and specificity than clinical syndrome and may improve the reliability of our findings.

An additional limitation stems from a potential causal intermediate. The degree to which an individual is immunocompromized due to HIV, which is associated with ART, may affect the risk of clinical malaria.(43, 45). Women assigned to NNRTI-based therapy in Trial 1 were more likely to experience virologic failure when compared to women randomized to LPV/r-based therapy.(CROI 2009 abstract) This reduced control of HIV disease could have an impact on our findings as immune status could be in intermediate on the hypothesized causal pathway between PI-based ART and incidence of malaria.

Finally, our study used a valid intent-to-treat analysis; though this is the gold standard statistically, information from an appropriately executed per-protocol analysis may have provided some additional information. Among the 346 women included in this ancillary analysis, 50 (14%) switched treatments due to virologic failure or toxicity associated with the regimen to which she was randomized.

Because a relatively small percentage of patients switched treatments and because fewer then 10% of the malaria episodes occurred after the participant had switched, we anticipate that in this study, the difference between findings from intent-to-treat and per-protocol analyses may have been modest.

4. Implications

This work is only the first step in understanding what, if any, the effect of PI-based therapy has on incidence of malaria in people infected with HIV. We feel confidant in our findings which were generated from longitudenal, clinical trials data. Our finding of no effect was similar across trials and was the same when we conducted a sensitivity analysis in which only laboratory-confirmed cases were considered to have experienced malaria.

Even given our confidence in our results, we know that there are still many unanswered questions and do not conclude that looking at the effect of HIV PIs on malaria in humans is not still worthwhile. Information on subclinical disease through the use of biomarkers will provide additional insight, as will looking at the clincal effect in children, who are at greater risk for malaria.

One important implication is that our findings suggest that there may be an insufficient overlap between results obtained in the laboratory and those generated *in vivo* to warrant additional laboratory studies that employ the same techniques.

C. Conclusions

Malaria remains a significant cause of morbidity and mortality. This dissertation explored two specific challenges to the successful treatment of malaria, two small attempts to address what remains a complex and important problem. We developed a novel way to estimate treatment efficacy and discovered that HIV PIs may not have antimalarial action in HIV-infected patients at risk of co-infection.

APPENDIX A: Human Subjects

The proposed research uses only simulated and de-identified data and is exempt from Institutional Review Board (IRB) review. A decision to this effect for Aim 2 was issued by the UNC-Chapel Hill School of Public Health (Public Health IRB # 09-0221, 2/6/2009).

APPENDIX B: Greenhouse *et al.*'s calculation of P_{match} and their true recrudescence formulas(5)

[Text and formulas adapted or taken directly from Greenhouse *et al.*(5)]

P_{match} (the estimated probability of a match occurring by chance) was calculated as follows: The relative probability of all possible combinations of the number of alleles in the post-treatment sample was estimated by multiplying together the frequency of each of the component alleles in the combination. Each possible combination was then compared to the alleles present in the pre-treatment sample to determine if there was at least one allelic match. P_{match} was calculated by taking the sum of the probabilities of combinations that matched the pretreatment sample and dividing by the sum of the probabilities of all combinations.

They estimated the number of true recrudescent infections by combining the following two equations.

$$n_{or} = n_{recru} + n_{new} \cdot \bar{P}_{match} \quad (1)$$

where n_{or} is the number of observed recrudescent infections, n_{recru} is the estimated number of true recrudescent infections, and n_{new} is the estimated number of true new infections, and

$$n_{rp} = n_{new} + n_{recru} \quad (2)$$

where n_{rp} is the number of recurrent-parasitemia samples. By solving equation 2 for n_{new}, substituting this into equation 1, and solving for n_{recru}, we arrive at equation 3:

$$n_{recru} = \frac{n_{or} - \bar{P}_{match} \cdot n_{rp}}{1 - \bar{P}_{match}}$$

APPENDIX C: Formulas from Kwiek *et al*.(21)

Participant-specific probability of a chance-match in indeterminate episodes with a single pre- and post-treatment shared band = $1 - (1 - y)^x$ where,

Y is the prevalence of the shared band and X is the number of variants in the recurrent parasitemia sample

Mean of participant-specific probabilities = Reinf

Number of indeterminate infections = Ind

Number of infections classified as recrudescent by genotyping = Recru

Estimated failure rate = [((1-Reinf) x Ind) + Recru]/Total number of participants

APPENDIX D: MATLAB® code adapted from code provided by Dr. Christina Burch, UNC – Chapel Hill.

```
param=[];

false_pos_rate=[];

for i = 1:10

reps = 10000;

argh = (i)*ones(reps,1);

pea = (argh/10);

init_infect1 = nbinrnd(argh,pea);

re_infect1 = nbinrnd(argh,pea);

param(i)=i;

test1 = init_infect1 == re_infect1;

false_pos_single(i) = sum(test1)/reps;
```

APPENDIX E: Format of the person-period dataset for discrete-time survival analysis

Study ID	Treatment	Time Period	D_8	D_{12}	D_{24}	D_{48}	Malaria
1	1	8	1	0	0	0	0
1	1	12	0	1	0	0	0
1	1	24	0	0	1	0	1
2	0	8	1	0	0	0	1
3	1	8	1	0	0	0	0
3	1	12	0	1	0	0	1
4	0	8	1	0	0	0	0
4	0	12	0	1	0	0	0
4	0	24	0	0	1	0	0
4	0	48	0	0	0	1	0

Treatment: LPV/r-based therapy = 1; NNRTI-based therapy = 0

Time period: number of weeks on study

D_j: Time period indicator variables

Malaria: Occurrence = 1; Non-occurrence = 0

APPENDIX F: Discrete-time survival analysis: Modeling the relationship between the population discrete-time hazard function and study treatment

$$\text{logit hazard}_{ij} = [\alpha_8 D_8 + \alpha_{12} D_{12} + \alpha_{24} D_{24} + \alpha_{48} D_{48}] + \beta_1(\text{Treatment})$$

$\alpha_8 D_8$ = baseline logit hazard function at week 8

$\alpha_{12} D_{12}$ = baseline logit hazard function at week 12

$\alpha_{24} D_{24}$ = baseline logit hazard function at week 24

$\alpha_{48} D_{48}$ = baseline logit hazard function at week 48

β_1 = slope parameter reflecting the effect of LPV/r-based therapy compared to NNRTI-based therapy on malaria

APPENDIX G. Numerical example of the adjustment of recrudescent infections

Subjects in trial, N = 100

Number of subjects with recrudescent infections as classified by PCR-correction = 15

Number of subjects with reinfections as classified by PCR-correction = 30

Y = Value sampled from distribution of the probabilities of false positives (in this example, Y = 0.17)

Z = Value sampled from distribution of the probabilities of false negatives (in this example, Z = 0.25)

Adjusted number of recrudescent infections = 15 – (15 x 0.17) + (30 x .25)

= 15 – 2.55 + 7.5 = 19.95

PCR-corrected cure rate = 1 – (15/100) = 0.85 or 85%

Cure rate adjusted for outcome misclassification = 1 – (19.95/100) = .8005 or 80.05%

APPENDIX H. Site and trial-specific survival curves for ACTG5208.

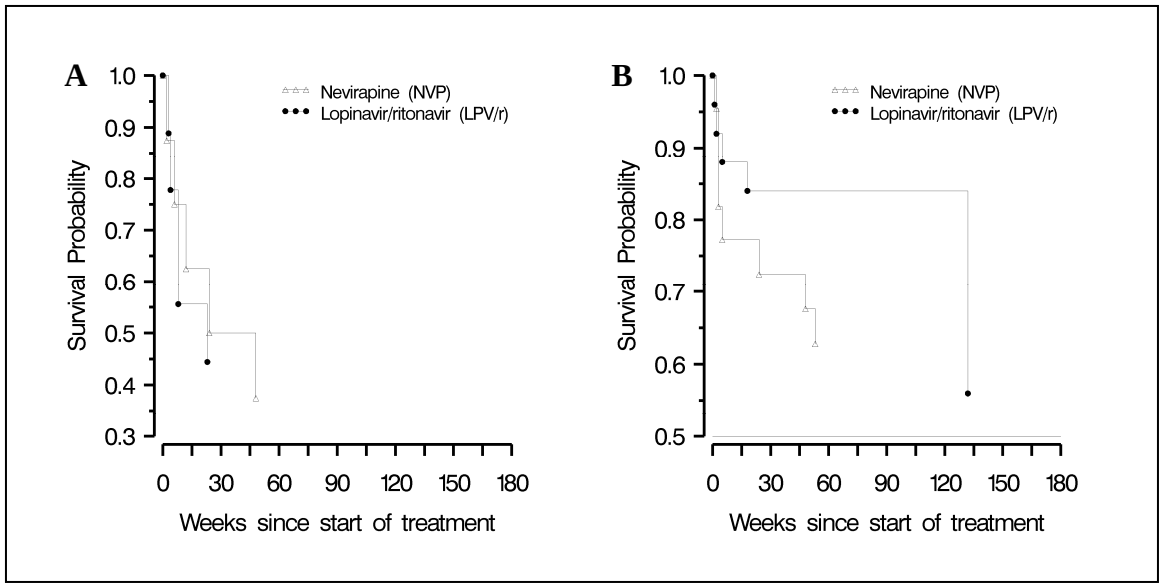

Figure 1. Survival curves for ACTG5208 participants in Eldoret, Kenya.

(A) Trial 1. (B) Trial 2.

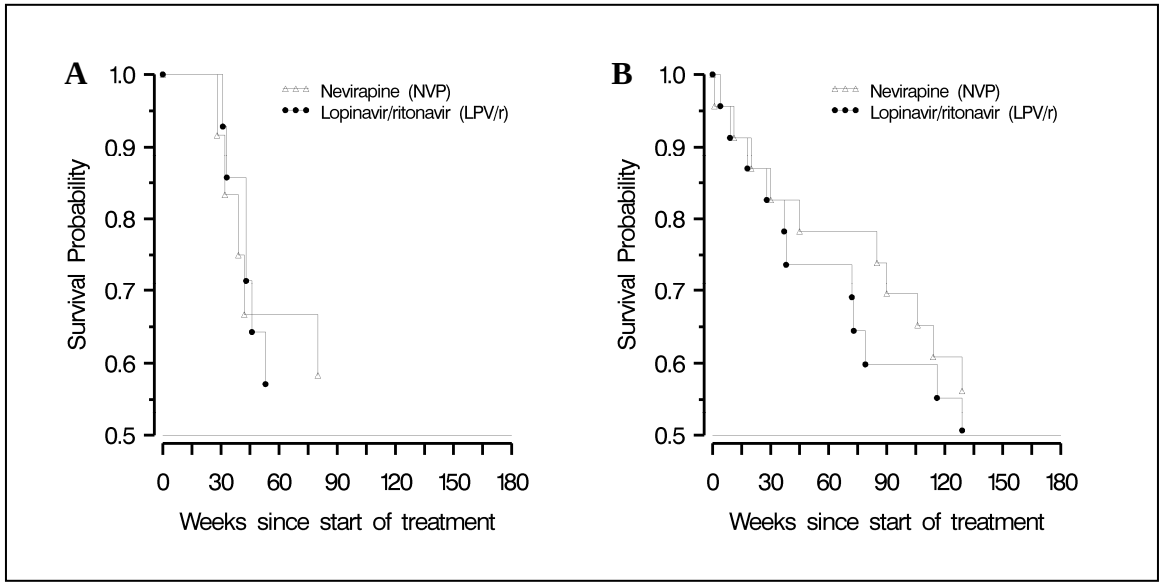

Figure 2. Survival curves for ACTG5208 participants in Kericho, Kenya.

(A) Trial 1. (B) Trial 2.

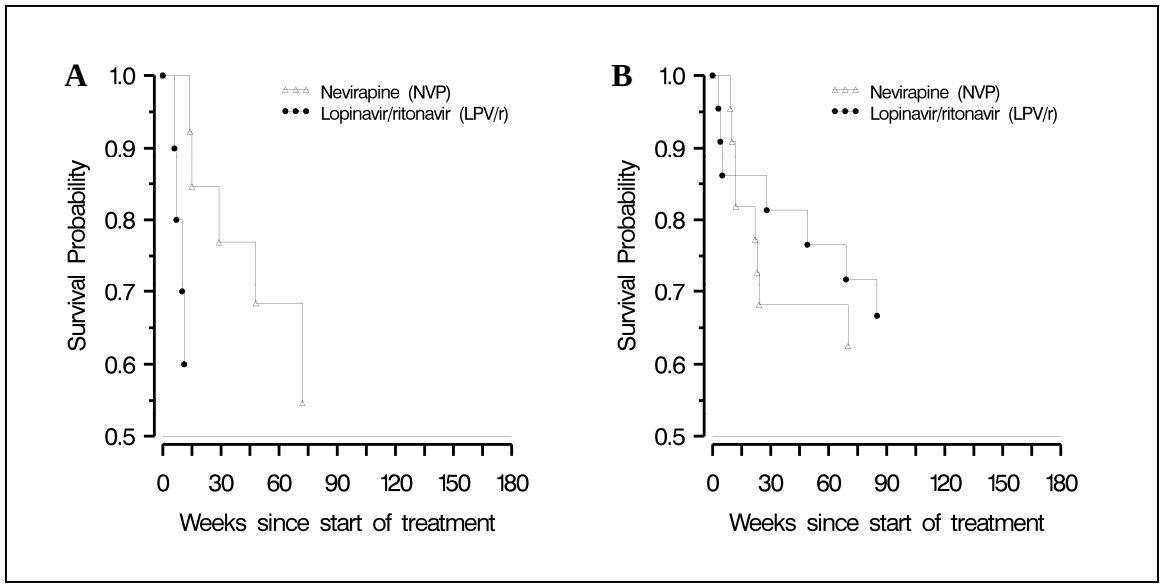

Figure 3. Survival curves for ACTG5208 participants in Lilongwe, Malawi.

(A) Trial 1. (B) Trial 2.

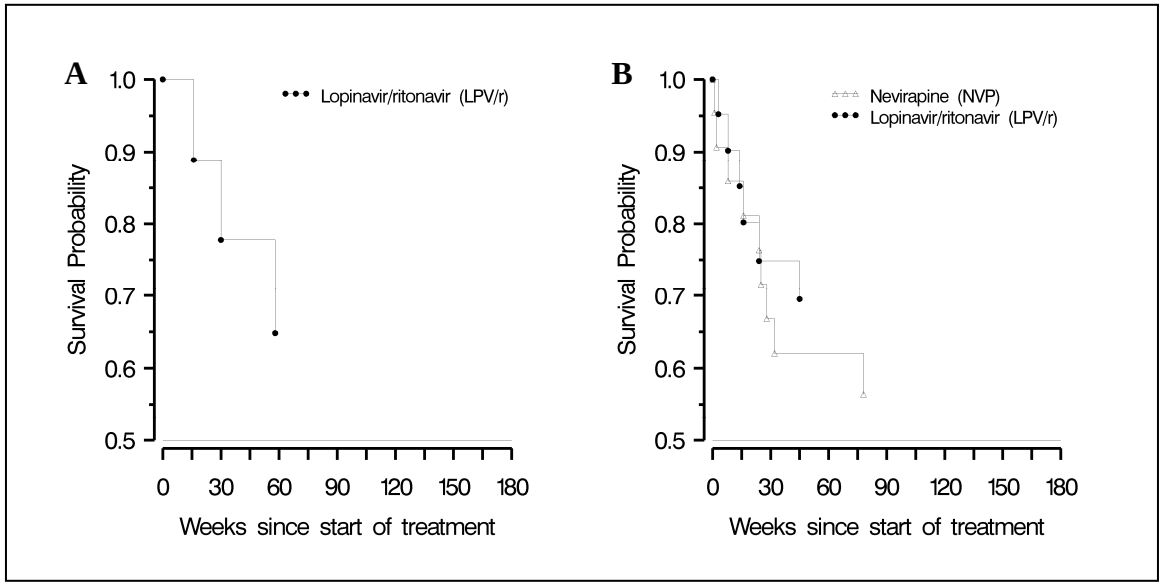

Figure 4. Survival curves for ACTG5208 participants in Kampala, Uganda.

(A) Trial 1. (B) Trial 2.

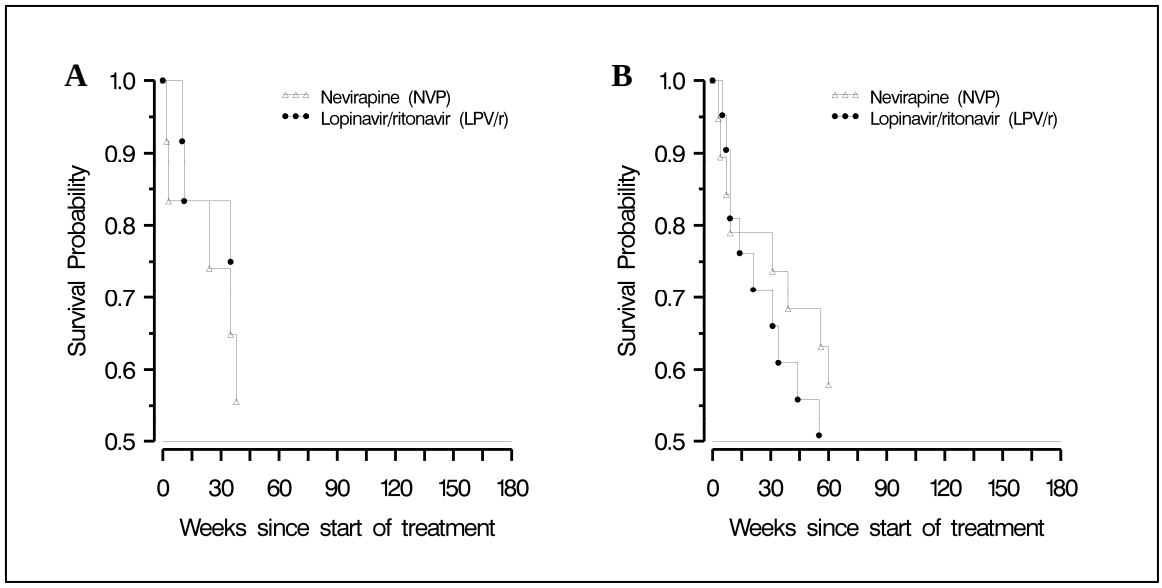

Figure 5. Survival curves for ACTG5208 participants in Lusaka, Zambia.

(A) Trial 1. (B) Trial 2

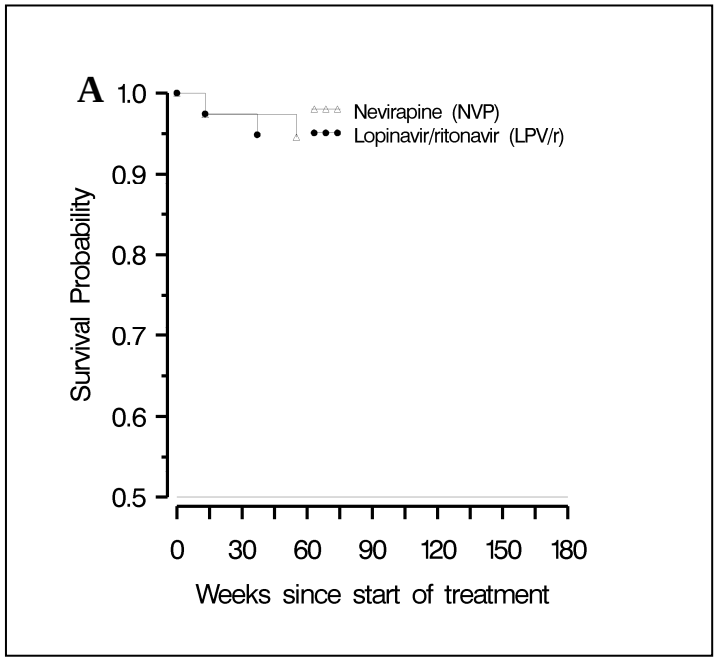

Figure 6. Survival curves for ACTG5208 participants in Harare, Zimbabwe.

(A) Trial 2. No participants experienced malaria in trial 1.

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