Monitoring and Deterring Drug-Resistant Malaria in the Era of Combination Therapy

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Abstract. As chloroquine and sulfadoxine-pyrimethamine (SP) are replaced by more effective artemisinin-based combination therapies (ACTs), strategies for monitoring (and, if possible, deterring) drug-resistant malaria must be updated and optimized. In vitro methods for measuring resistance will be critical for confirming and characterizing resistance to ACTs. Molecular markers are useful for tracking the emergence and dissemination of resistance and guiding treatment policy where resistance is low or moderate. Genomic approaches may help identify molecular markers for resistance to artemisinins and their partner drugs. Studies of reported ACT treatment failure should include assessing factors other than resistance that affect efficacy, including pharmacokinetics. Longitudinal clinical trials are particularly useful for comparing the benefits and risks of repeated treatment in high transmission settings. The malaria research and control community should not fail to exploit this opportunity to apply the lessons of the last 50 years to extend the useful therapeutic lives of ACTs.

INTRODUCTION

The landscape of antimalarial therapy is changing. With new multilateral support for artemisinin-based combination therapies (ACTs), highly efficacious alternatives are becoming available to replace less effective drugs (chloroquine and sulfadoxine-pyrimethamine [SP]) that are still used widely despite their impaired efficacy. New drugs and treatment strategies will save many lives every day. Combination therapy presents new challenges for monitoring resistance and efficacy, as well as new prospects for deterring drug resistance. This review will explore the emerging and complex issues surrounding monitoring of antimalarial drug resistance in the era of combination treatment of Plasmodium falciparum malaria. We suggest that monitoring should go beyond passive documentation of the success or failure of antimalarial treatment. The influx of drugs, infrastructure, support, and enthusiasm that is accompanying the transition to combination therapies presents opportunities to gain new understanding of how resistance emerges and spreads. This knowledge will in turn inform strategies for deterring, delaying, and containing resistance.

IN VITRO SUSCEPTIBILITY ASSESSMENT

Methods for measuring parasite growth in vitro in the presence of increasing drug concentrations were developed for culture-adapted malaria parasites in controlled laboratory settings. In vitro tests determine the intrinsic susceptibility of the parasite to drugs without the confounding effects of host factors such as immunity or pharmacokinetics, and thus, provide a direct, quantitative assessment of drug resistance.

Limitations of in vitro tests. Most discussions about strategies for monitoring antimalarial drug efficacy start with the limitations of in vitro methods. Adapting these methods for field testing of clinical isolates has been challenging for reasons reviewed elsewhere. In several cases, clinical trials evaluating drugs to which good in vitro susceptibility had been documented found unexpectedly high rates of treatment failure. Factors other than parasite resistance can contribute to the failure of clinical isolates to grow in vitro, leading to false alarms for emerging resistance. The results of rapid in vitro tests done in the field must be viewed with caution until they are confirmed by repeated testing under carefully controlled conditions in the laboratory. This requires preservation and culture adaptation of field isolates.

At present, a clear definition of in vitro resistance to the artemisinin derivatives is not available. Although decreased in vitro susceptibility to the artemisinins has been reported in small numbers of field isolates, bona fide, clinically significant resistance to artemisinin drugs has not been convincingly documented, and the significance of the variations that have been described in susceptibility to artemisinins in vitro in some isolates is unknown. Understanding the clinical relevance of in vitro artemisinin susceptibility is further complicated by the fact that these drugs are almost always used in combination with other drugs.

Critical role for in vitro tests in the ACT era. Despite these limitations, in vitro assays are increasingly important in the era of ACTs because of the inability to rely on molecular methods for monitoring resistance and the absence to date of clinically significant resistance to the artemisinins. The early stages of parasite resistance to individual drugs used in combination therapy regimens may not be clinically apparent because of the action of the partner drug(s). Clinical studies to monitor efficacy may thus be relatively insensitive for heralding the impending failure of drug combinations. While candidate molecular markers for resistance to artemisinins are being studied and molecular correlates of responses to several ACT partner drugs have been described, validated molecular markers for clinical ACT treatment failure are not available and will not become available unless true clinical resistance to the artemisinins appears, for the simple reason that it is not possible to validate a marker for a phenomenon that does not yet exist. For these reasons, in vitro tests are the front line of resistance monitoring for artemisinins and ACTs.

As an early warning system, routine ongoing in vitro monitoring of resistance to artemisinins and ACT partner drugs should be performed, particularly in parts of the world where ACTs have been used the most. When reports of clinical treatment failures of ACTs arise, or where clinical trials show...
higher than expected rates of treatment failure, rigorous in vitro testing with cryopreservation of parasite isolates should be done in a targeted fashion, modeled after outbreak studies performed by epidemic intelligence services (Figure 1). In a stepwise fashion, factors including resistance to individual partner drugs, pharmacological parameters, and host immunity can be assessed for their potential contributions to treatment failure. If true resistance to artemisinins or partner drugs is documented, genomic analyses can be done to identify molecular markers for resistance, as described below.

Clinical treatment failure can occur for reasons other than intrinsic parasite resistance. If clinical failures of ACTs are documented, careful in vitro testing can help to determine if the failures were caused by resistance or to other causes, such as poor drug quality, pharmacokinetic variability, or drug interactions. Aggressive study of possible resistance to artemisinins and their partner drugs should be a high priority as these drugs are rolled out. Capacity in malaria endemic areas must be expanded to enable reliable, high-quality in vitro susceptibility testing to occur at or near sentinel sites. If definitive in vitro testing is not done expeditiously, repeated unsubstantiated reports of clinical resistance may result in a “boy who cried wolf” situation, in which the eventual emergence of true resistance goes unrecognized until the opportunity to contain it has passed.

MOLECULAR MARKERS FOR DRUG RESISTANCE

Molecular markers for resistance to monotherapies. In the late 20th century, candidate molecular markers for antimalarial drug resistance were identified by cloning and sequencing parasite genes homologous to those known to mediate resistance in other organisms and/or by using reverse genetics approaches to analyze the progeny of genetic crosses between sensitive and resistant parasites. Based on differences in DNA sequence between sensitive and resistant parasite clones, point mutations, variable length repeat sequences, and/or differences in copy number were evaluated for their association with in vitro resistance phenotypes. In some cases, the link between molecular markers and in vitro resistance was proved (or disproved) in genetic transformation studies in which sensitive parasites were rendered resistant or vice-versa by precise substitutions of genetic sequences in parasite clones maintained in laboratory culture or in model systems such as yeast.

Finally, molecular markers for resistance were assessed in ecological studies and clinical trials to establish their association with, and ability to predict, treatment failure in vivo. This process of identifying and evaluating molecular markers of resistance has been painstakingly slow, and reasonably reliable methods for using molecular markers to monitor chloroquine and SP resistance were established only after resistance to these drugs was so widespread that the information provided by molecular surveys was, with some exceptions described below, mainly of academic interest and of little immediate relevance to antimalarial treatment policies.

Validation and application of molecular markers for chloroquine and SP resistance. Because factors other than intrinsic parasite resistance contribute to clinical and parasitologic outcomes of antimalarial drug treatment, establishing the predictive value of molecular markers for treatment outcomes has been challenging. Even for markers with virtually absolute correlation between genotype and in vitro phenotype, other factors including acquired immunity and pharmacokinetic parameters affect the clearance of drug-resistant parasites. In an attempt to use resistance markers to predict treatment outcomes, immunity, the most important contributor to clearance of resistant parasites in areas of high malaria endemicity, was accounted for by controlling for age in a simple model using the prevalence of molecular markers to predict treatment failure rates. In this model, the genotype-failure index (GFI) is the ratio of the prevalence of resistant genotypes to the rate of treatment failure in a population. Once established and adjusted for age, it was suggested that

![Figure 1. Algorithm for studying reports of resistance to ACTs. PK, pharmacokinetics.](image-url)
GFIs could be used to predict the treatment failure rate in a population based on simple measurements of the prevalence of a resistance marker in that population. This approach is best suited to populations with stable malaria endemicity so that a GFI calculated for a specific population at one point in time remains valid for predicting failure rates in that population as the prevalence of resistance markers changes over time.

The GFI model was developed and assessed as a tool for predicting chloroquine treatment failure rates at four sites in Mali, and has since been applied in several countries in Africa. Table 1 summarizes results of published papers that both cite the paper proposing the GFI model\(^{27}\) and include data from which GFIs can be calculated directly. As expected, at sites where the prevalence of the chloroquine resistance marker approached 100%, GFIs varied widely. This is because when the resistant genotype approaches fixation in a population, factors other than resistance must determine treatment outcomes.\(^{36}\) Thus, the GFI model is both most useful and most relevant where resistance is still low to moderate,\(^{36}\) but even so, a very high prevalence of molecular markers for resistance is a reliable if imprecise indication that treatment failure rates are unacceptably high. In settings with treatment failure rates of 50% or higher, tools to predict precisely how far efficacy has fallen below acceptable thresholds are not needed. Indeed, continuing large efficacy trials of drugs where they are known to be poorly efficacious is ethically questionable.\(^{39}\) Especially now that more effective ACTs are becoming widely available, such trials should not be repeated.

Where chloroquine treatment failure rates were < 50%, which is to say at sites where a molecular tool for predicting clinical outcomes may provide information useful for guiding treatment policy, GFIs were close to 2 at most sites. This allows straightforward interpretation of molecular surveys for the chloroquine resistance marker: the predicted rate of treatment failure is equal to about one half of the prevalence of the marker.

Several studies have applied the GFI model to predict SP failure rates\(^{26,35,36,40}\) in Tanzania systematically calculated GFIs at five sites using the DHFR triple mutant as the genotype to predict SP treatment failure at 14 days. At four sites with SP treatment failure rates ranging from 7% to 14%, age-adjusted GFIs were between 2.0 and 2.1. At one site with an 80% prevalence of the resistance marker, the GFI was 3.4, consistent with the idea that GFIs are unreliable when the prevalence of the marker approaches fixation in the population. Studies done in Malawi, Uganda, and Nigeria also had GFIs for SP that were very close to 2, despite using different sets of markers for SP resistance and different definitions of treatment failure, suggesting that GFIs are a robust tool for predicting SP treatment failure rates in settings of low to moderate resistance.

The use of molecular markers for predicting resistance could be further improved with more sophisticated multivariate models that take into account pharmacokinetic, host genetics, and other factors that contribute to treatment outcome, although such comprehensive models would be impractical for routine surveillance of resistance. Additional refinements that might improve the GFI model include studies confirming that different sampling strategies such as cross-sectional population surveys and collection of samples at sick clinic visits yield comparable estimates of genotype prevalence and testing the suggestion to use calculated allele frequencies rather than simple genotype prevalence.\(^{41}\)

This GFI model has been used to guide malaria treatment in an outbreak of malaria in Mali, where civil unrest precluded other means of assessing drug efficacy\(^{42}\) and to guide national treatment policy in Tanzania.\(^{36}\) In addition to being used to guide treatment policies, resistance markers have also predicted the return of susceptible parasites after the removal of drug pressure. In Malawi, a decrease in the prevalence of the molecular marker for chloroquine resistance was detected soon after the country switched from chloroquine to SP for the first line therapy of malaria because of very high rates of clinical failure. Within 8 years, parasites with the resistance marker were undetectable.\(^{43}\) A clinical trial confirmed that chloroquine again had excellent clinical efficacy, 12 years after it was removed from use.\(^{44}\) As ACTs replace chloroquine and SP, monitoring the prevalence of molecular markers may signal a time to consider reintroducing these drugs as components of combination therapy or in targeted interventions such as intermittent preventive treatment.

**Molecular markers for resistance to ACTs in the genomic era.** In the search for molecular markers of drug-resistant malaria, the low-hanging fruit has been picked. In the cases of chloroquine and the antifolates, investigators were fortunate that their heroic efforts using reverse genetics and candidate

### Table 1

Published drug efficacy studies directly or indirectly assessing the GFI model for using molecular markers for drug-resistant malaria to predict treatment outcomes

<table>
<thead>
<tr>
<th>Country</th>
<th>No. sites</th>
<th>Drug</th>
<th>Resistant genotype</th>
<th>Prevalence of genotype</th>
<th>Treatment failure rate GFI</th>
<th>Age adjusted</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mali</td>
<td>4</td>
<td>Chloroquine</td>
<td>PICRT T76</td>
<td>13–28%</td>
<td>43–66%</td>
<td>1.6–2.7</td>
<td>Yes</td>
</tr>
<tr>
<td>Ghana</td>
<td>1</td>
<td>Chloroquine</td>
<td>PICRT T76</td>
<td>81%</td>
<td>58%</td>
<td>1.4</td>
<td>NA</td>
</tr>
<tr>
<td>Congo</td>
<td>1</td>
<td>Chloroquine</td>
<td>PICRT T76</td>
<td>100%</td>
<td>96%</td>
<td>1.04</td>
<td>NA</td>
</tr>
<tr>
<td>Tanzania</td>
<td>1</td>
<td>Chloroquine</td>
<td>PICRT T76</td>
<td>100%</td>
<td>51%</td>
<td>2.0</td>
<td>No</td>
</tr>
<tr>
<td>Sudan</td>
<td>1</td>
<td>Chloroquine</td>
<td>PICRT T76</td>
<td>100%</td>
<td>61%</td>
<td>1.6</td>
<td>No</td>
</tr>
<tr>
<td>Burkina Faso</td>
<td>4</td>
<td>Chloroquine</td>
<td>PICRT T76</td>
<td>22–65%</td>
<td>15–37%</td>
<td>1.1–3.0</td>
<td>No</td>
</tr>
<tr>
<td>Malawi</td>
<td>1</td>
<td>SP</td>
<td>DHFR 59 + DHPS 540</td>
<td>65%</td>
<td>30%</td>
<td>2.2</td>
<td>NA</td>
</tr>
<tr>
<td>Tanzania</td>
<td>5</td>
<td>SP</td>
<td>DHFR triple mutant</td>
<td>19–80%</td>
<td>7–24%</td>
<td>2.0–3.4</td>
<td>Yes</td>
</tr>
<tr>
<td>Uganda</td>
<td>1</td>
<td>SP</td>
<td>DHFR 59 + DHPS 540</td>
<td>61%</td>
<td>33%</td>
<td>1.9</td>
<td>NA</td>
</tr>
<tr>
<td>Nigeria</td>
<td>1</td>
<td>SP</td>
<td>DHFR triple mutant</td>
<td>46%</td>
<td>25%</td>
<td>1.8</td>
<td>NA</td>
</tr>
</tbody>
</table>

\(\text{PICRT, } P. falciparum \text{ chloroquine resistance transporter; DHFR, dihydrofolate reductase; DHPS, dihydropyroate synthase; ETF, early treatment failure; LCF, late clinical failure; LPF, late parasitologic failure; NA, not applicable; SP, sulfadoxine pyrimethamine.}\)
gene approaches eventually paid off with the identification of key resistance genes, which, even if they are not the sole genetic contributors to resistance, are clearly its primary determinants. In the ACT era, we cannot count on being so fortunate again. Candidate gene approaches based on non-malaria homologs or on suspected mechanisms of drug action have been used to study genetic determinants of resistance to drugs included in ACTs. In an example of the homolog candidate gene approach, in vitro and clinical evidence suggests that increased pfmdr1 copy number is associated with resistance to mefloquine, lumefantrine, and artemisinin, as well as other antimalarial drugs. Based on studies showing that artemisinins inhibit Ca\(^{2+}\) ATPase, polymorphisms in PfATPase\(_6\) have been measured in isolates tested for in vitro susceptibility to artemisinin derivatives in rapid in vitro field tests. PfATPase\(_6\) mutations were reported to be associated with artesunate resistance in a small number of isolates from French Guiana but not in samples from Senegal or Cambodia. Although such studies are useful for hypothesis generation, carefully designed prospective studies with adequate statistical power and population genetics analyses that account for regional allelic diversity are needed for valid assessments of putative molecular markers for resistance to the artemisinins. More importantly, the candidate gene approach relies on what may be overly optimistic hope that a single gene can be identified that encodes resistance to artemisinins. If, as seems likely, resistance to artemisinins and their partner drugs are mediated by multiple genes, a coordinated global consortium using genomic tools to identify molecular markers of ACT resistance is more likely to be profitable than an iterative gene-by-gene candidate gene approach with rival laboratories each championing their favorite candidate.

The ability to study the molecular basis of antimalarial drug resistance has been revolutionized by the sequencing of the \textit{P. falciparum} genome. With the genome fully sequenced and being interrogated with a variety of approaches, the opportunity exists to accelerate the process of identifying molecular markers for resistance to the drugs used in ACTs. The challenge for genomic scientists, molecular parasitologists, and clinical translational researchers is to link cutting edge genomic technology with clinical research and public health surveillance in a worldwide effort to expeditiously identify molecular markers of resistance to the new ACTs. We need to be poised to aggressively study reports of resistance, confirm it with in vitro assays, and bring genomic tools to bear to elucidate the mechanisms and identify candidate molecular markers of artemisinin resistance (Figure 1).

Using microsatellites and single nucleotide polymorphisms (SNPs), genome-wide maps of quantitative trait loci associated with resistance to ACTs can be created and used to detect regions of the \textit{P. falciparum} genome that are under selection by ACT treatment. This approach may provide clues to the molecular bases of resistance to ACTs even before frank clinical resistance arises, but its real promise lies in focused application to parasite isolates corresponding to cases of clinical treatment failure confirmed to represent resistance by careful, reproducible in vitro testing. Molecular evolutionary approaches developed to measure selection in other genomes, such as measures of extended haplotype homozygosity, may also detect areas of the \textit{P. falciparum} genome that are under recent positive selection by ACTs. Ultimately, it will be necessary to resequence large numbers of samples from initial infections that cleared or failed to clear with ACT treatment and from post-treatment recrudescence infections, to identify specific polymorphisms associated with resistance to and selected by the ACTs. Because gene expression may also contribute to resistance, DNA microarray analyses of gene expression will also be needed. Such direct application of advanced genomic technologies to analyze clinical samples from field trials in developing countries to develop new tools for public health surveillance will be unprecedented. Many technical and analytical problems will have to be solved for this approach to work, requiring close collaboration between genomic scientists and researchers in the field, but the rationale for solving these problems could hardly be stronger. Resistance to ACTs must be detected and confirmed as soon as it emerges, so that tools to monitor it can be developed, validated, and applied in time to make rational policies and design and deploy drug combination technologies to deter and contain this resistance.

PHARMACOKINETICS

In clinical assessments of drug efficacy, apparent drug failures interpreted as clinical resistance may reflect the bioavailability and metabolism of the drug rather than parasite resistance. For example, the bioavailability of lumefantrine is highly variable and is influenced by a number of factors, including the amount of fat consumed at the time of administration. Lumefantrine drug level is the strongest predictor of the outcome of treatment with one of the leading ACTs, lumefantrine-artemether. Alternatively, higher drug levels may be associated with increased rates of clearance of drug-resistant parasites. Although it may be neither practical nor necessary to include measures of drug levels in all drug efficacy trials, pharmacokinetic analyses are a critical element in studying and confirming reports of resistance to ACTs (Figure 1). Differences in pharmacokinetic parameters may explain a lack of correlation between in vitro, molecular, and clinical measures of resistance. In particular, if therapeutic drug levels are not achieved or maintained for an adequate time, treatment failures may turn out to have nothing to do with resistance.

The possibility of pharmacokinetic interactions between antimalarial and other drugs must also be considered as ACTs are rolled out, although fortunately, clinically significant interactions have not yet been reported for common antimalarial drugs. In malaria-endemic regions, concomitant treatment is common for multiple illnesses including acute bacterial infections, HIV, and tuberculosis. The role of pharmacokinetic interactions in responses to treatment with antimalarial drugs is only beginning to be explored. If important drug–drug interactions are identified, it will at a minimum be necessary to record drug use in clinical assessments of in vivo resistance.

CLINICAL ASSESSMENT OF DRUG EFFICACY

Different methods are used to measure antimalarial drug efficacy for different purposes. For ongoing monitoring of the therapeutic efficacy of standard first- and second-line drugs, most malaria control programs and malaria researchers have relied on protocols published by the World Health Organiz-
tion. More rigorous controlled clinical trials are used to assess the safety and efficacy of investigational new drugs and drug combinations. Longitudinal clinical trials represent a relatively new tool that is particularly well suited for comparing treatment regimens with similar high efficacy as measured in standard trials to select those with the greatest health benefit in particular settings.

**In vivo protocols for monitoring therapeutic efficacy.** The standard in vivo protocols used to monitor antimalarial drug resistance have been updated repeatedly by expert panels convened by the World Health Organization. A 1973 protocol relied strictly on the presence or absence of parasites at intervals after treatment to classify infections as sensitive or as resistant at the RI, RII, or RIII levels.57 A protocol published in 1996 did not consider recurrent parasitemia after post-treatment Day 3 to constitute treatment failure unless it was accompanied by a documented fever, resulting in many cases of RI and RII resistance being classified as “adequate clinical responses” and not treatment failures.58 A cynic might wonder whether this change reflected recognition that resistance to chloroquine and SP had resulted in unacceptably low efficacy and that, lacking more efficacious alternatives, the only way to improve the situation was to redefine treatment failure to raise point estimates of efficacy. The next iteration, published in 2003, in turn reflected new optimism based on the introduction of ACTs in southeast Asia, as well as increased appreciation of the potential deleterious effects of chronic malaria infection, and returned to a definition of efficacy, “adequate clinical and parasitological response,” that required the resolution of parasitemia.59 Present discussions focus on the role of genotyping to distinguish reinfection from recrudescence and on whether the duration of the post-treatment parasite-free period should be extended to allow more sensitive detection of resistance manifested by increased rates of late treatment failure.60 The evolution of the tool used to monitor in vivo efficacy of antimalarial drugs thus continues to reflect the state of affairs of malaria treatment—as the first line increasingly shifts to highly efficacious ACTs for which significant resistance has not yet arisen, the definition of efficacy is changing to permit detection of the most subtle, early signs of resistance.

**Controlled clinical trials.** Controlled clinical trials are used to assess the safety and efficacy of drugs and drug combinations being considered for regulatory registration and to compare treatment regimens in preparation for changes in policy recommendations. These trials are distinguished from standard in vivo therapeutic efficacy protocols by the inclusion of different study arms comparing treatment regimens, by the collection of additional safety data, and in recent years, by required adherence to rigorous Good Clinical Practices (GCP).

The requirement that regulatory clinical trials of malaria drugs be conducted in compliance with GCP has been described as so burdensome as to impede important clinical research in poor countries.61 Phase I, II, and III clinical trials of investigational new drugs and vaccines on the path to regulatory registration can and should be conducted in compliance with GCP, and this is now regularly done in the poorest African countries. Several malaria vaccine trials done under GCP in countries like Mozambique, Mali, and Kenya have been published,62-64 and dozens of GCP-compliant malaria drug trials in developing countries are listed in clinical trial registries on the internet. Following the strict standards of GCP and reporting all adverse events in a trial assures the sponsors and the public of the validity and completeness of the data, but the level of regulatory oversight and monitoring (which are not the same thing as GCP) should be appropriate for the purpose of the study; a study of a new drug requires more detailed safety review than one of a drug whose safety profile is already well established. Indiscriminate application of the same maximum degree of regulatory oversight and safety monitoring to all drug studies will curtail important research needed to monitor the implementation of ACTs.

Some studies of drug combinations have been conducted using a factorial design, permitting assessment of the specific contribution of individual partner drugs to the outcome of treatment with combinations of drugs. For example, a study in Uganda compared SP alone with SP combined with either artesunate or amodiaquine, allowing straightforward determination of the benefit of each of the partner drugs.65 Since the adoption of ACTs in Southeast Asia, such studies have largely been replaced by comparisons of combinations without contemporaneous assessment of individual partner drugs. Examples include comparisons of mefloquine-artesunate to artemether-lumefantrine in Thailand66 and to dihydroartemisinin-piperaine in Vietnam.67 In these trials, it difficult if not impossible to determine whether differences in efficacy are caused by differences in the performance of the short-acting artemisinins, the long-acting partner drugs, or both. This limitation of clinical trials of ACTs again highlights the critical role for in vitro assays in studying possible resistance and the need to develop reliable molecular markers for resistance to ACTs.

**Longitudinal trials.** In longitudinal trials, participants are enrolled at the time of presentation with acute illness caused by uncomplicated malaria and followed prospectively for a specified time, usually 1 year. Participants are randomized to receive a specified treatment regimen at the time of enrollment and get the same treatment every time they develop clinical malaria during the study period. The most compelling argument for longitudinal trials is that they provide a realistic estimation of the true public health benefit of the study treatments, something that studies measuring the efficacy of single treatment episodes cannot do. People in many malaria-endemic areas develop clinical malaria up to several times each year, and treatment policy in these areas should ideally be based on sustained efficacy over time. The toxicity of drugs may also accumulate with repeated administration, so assessment of safety with dosing intervals that reflect the actual use of the drug are more informative than single-episode studies. Longitudinal trials may also reveal seasonal differences in how well drugs perform as transmission levels wax and wane. Where transmission is very low and few people experience more than one malaria episode per year, the usefulness of longitudinal trials is limited to their ability to detect very late recrudescences.

The primary outcome measure of longitudinal trials is the cumulative incidence of clinical episodes of malaria needing treatment. Secondary outcome measures include adequate clinical and parasitological response at 28 days or at any desired interval, for the initial episode as well as for subsequent episodes, to measure waning efficacy over time; incidence of new infections and recrudescent infections; incidence of anemia; incidence of severe malaria; and repeated measures of
safety, which can include laboratory measures of renal and hepatic function, neurologic abnormalities, or other adverse events. More global measures of health in the different arms can be recorded such as hemoglobin level at the end of the study and pediatric growth and development. These measures further reflect the impact of the treatment regimens on overall health in malaria-endemic regions.

Associations of molecular markers with treatment outcomes and selection for resistance genotypes can be measured in longitudinal trials, and this study design is particularly well suited for pharmacokinetic studies because of the ability to measure drug levels at any time before or after treatment. Longitudinal trials also allow for direct comparison of the benefits of drugs with differing pharmacokinetic characteristics. In areas of high transmission, the prophylactic benefit provided by a long-acting drug must be weighed against the increased selection of drug-resistant parasites as new infections are acquired during the extended period when subtherapeutic drug levels are present in the blood.

The first longitudinal trial to study the effects of drugs with very different pharmacokinetic profiles was a double-blind, placebo-controlled trial of SP and chlorproguanil-dapsone in Kenya and Malawi. These similar antifolate combinations are distinguished by the much shorter half-life of chlorproguanil-dapsone (CD) and its higher efficacy in Africa, where it has been shown to be highly efficacious even against infections resistant to SP. The study randomized children to receive either CD or SP for all uncomplicated falciparum malaria episodes over the course of a year and compared the cumulative number of treatment episodes and rates of treatment failure and anemia. In Malawi, CD had an efficacy of 95% compared with only 80% for SP. However, there were no differences between the treatment groups in the incidence of uncomplicated malaria episodes, anemia, or severe malaria, suggesting that the disadvantage of lacking a long prophylactic effect caused by CD’s short half-life was offset by its better efficacy and possibly by less selection for antifolate-resistant parasites. Researchers in Uganda have also used this method to show the superiority of SP plus amodiaquine over SP plus artesunate by comparing incidence density of malaria treatment episodes. The “best” drug combinations to adopt as treatment policy should be chosen based on their efficacy and safety over time and their ability to decrease the overall burden of clinical malaria in the population.

Genotyping to distinguish recrudescence from new infections. In all types of clinical studies of antimalarial drug efficacy, there has been a recent emphasis on distinguishing between new and recrudescent infection after treatment. The strategy for differentiating these outcomes has been to genotype highly polymorphic regions of the parasite genome to determine if the parasite “strains” causing the post-treatment infection are the same as (indicating a recrudescent infection) or different from (indicating a new infection) those present at the time of treatment. The most common method used employs polymerase chain reaction (PCR) to amplify specific regions of polymorphic genes encoding the antigens merozoite surface protein-1 (MSP-1), MSP-2, and glutamine-rich protein (GLURP). These methods for strain-specific genotyping have been criticized. The selected targets are not ideal because they are under immune selection pressure, which might bias the distribution of polymorphic forms of these antigens in different populations and age groups. Any genotyping strategy has a limit of detection and may fail to detect minority populations present initially at levels below this threshold. When these (possibly drug-resistant) minority parasites recrudesce later, they will be incorrectly labeled as a new infection and not included in “PCR-corrected” efficacy estimates. On the other hand, limited discriminatory power, particularly in less genetically diverse parasite populations, will lead to the mistaken labeling of new infections as recrudescent.

The use of microsatellites instead of genes encoding polymorphic antigens to distinguish recrudescence from reinfection may resolve some of these shortcomings. Microsatellites are short, variable-length, non-coding sequences of DNA that are presumably not under natural immune selection. Genotyping using microsatellites can detect higher genetic diversity than methods using MSP-1, MSP-2, and GLURP. Microsatellite methods have increased sensitivity for detecting new infections and may be less likely to misclassify new infections as recrudesences in polyclonal infections.

Genotyping to distinguish between recrudescence and new infections may not be necessary in all studies of antimalarial efficacy, depending on the purpose of the study. Before ACTs, all post-treatment recurrent infections were considered to represent resistance. In most cases this was the correct interpretation, because common drugs including chloroquine, SP, and mefloquine have such long durations of action that any parasites detected in the 2–4 weeks after treatment are surviving exposure to drug concentrations that should inhibit the growth of sensitive parasites. Thus, for studies intended to detect emerging resistance after treatment with long-acting drugs, it may be irrelevant whether recurrent infections are caused by recrudescence or new infection. For studies designed to inform treatment policy decisions, the incidence of malaria treatment episodes and the risk of complications such as anemia and severe malaria are the outcomes of interest, and again differentiating recrudescence from reinfection may not be relevant—drugs that reduce post-treatment infections and life-threatening complications are better than drugs that do not, whether these infections are caused by new or old parasites.

As ACTs began to be compared with each other and to monotherapies in high-transmission settings, it was felt to be important to distinguish between failure to clear primary infections and failure to prevent new infections in the post-treatment period. This distinction is important for uniform assessment of efficacy of new drugs across regions with varying intensity of malaria transmission and may be required for regulatory approval. Essentially, the idea is that drugs with shorter durations of action should not be “blamed” for high rates of late treatment failure caused by new infections, even though from the point of view of the patient experiencing the recurrent infection it may matter little whether the parasites are old or new. In fact, little is known about the relative risks and benefits of recrudescence versus reinfection after antimalarial treatment, and it is conceivable that new infections carry a higher risk of causing illness than do recrudesences of parasites to which an immune response has already been developed. If this is the case, in high-transmission areas, a longer-acting drug with “leaky” initial efficacy might be preferable to a highly efficacious ACT with a short duration of action, even though the radical cure provided by the latter is clearly more beneficial in low-transmission settings where the risk of new
infections is low. To our knowledge, no published studies address the question of whether new and recrudescent infections carry different risks.

Genotyping recurrent infections in longitudinal trials should make it possible to compare the time to next clinical episode after post-treatment recrudescences and reinfections. It would be valuable to do these analyses in trials of various drugs in populations with different levels of acquired immunity. If clinically meaningful differences are shown for post-treatment recrudescence and reinfection, this would provide a stronger rationale for including these genotyping analyses in drug efficacy trials. Until more is known about the relative risks of different types of post-treatment infections, from a public health perspective, all post-treatment infections should be considered “treatment failure” in recognition that malaria parasitemia is an undesirable outcome, regardless of the origin of the infection. Although “PCR-corrected” efficacy may allow for easier comparisons between Africa, South America, and Asia, in our view, it is not the best way to measure efficacy in high-transmission settings.

Distinguishing recrudescence from new infection in conjunction with measurement of molecular markers for resistance may help to understand the propagation and spread of resistance. Treatment failure with recrudescent parasites allows for the persistent transmission of resistant parasites. New infections may occur either in the face of inhibitory drug levels, representing resistance, or they may occur when drug levels are low or absent in the blood, representing parasites that are fully susceptible to the drugs being used. Describing the dynamics of selection of resistance and defining the “selection windows” after treatment with different drug combinations will aid in the rational design of new combinations of drugs based on consideration of their pharmacokinetic and pharmacodynamic properties.

SUMMARY

Fatalism and fear prevented many countries from letting go of chloroquine, despite its obvious failure. Now, after years of continued use of failing drugs in many countries, a consensus has formed to replace them with effective combination therapies. ACTs, with their rapid action and excellent efficacy, are being embraced by policy makers throughout Africa. With multisectoral support for procurement and infrastructure, the plan is to distribute ACTs widely. However, the current generation of ACTs will not maintain their efficacy indefinitely. Researchers and malaria control workers of all stripes must join efforts to apply in vitro, molecular, genomic, pharmacokinetic, and clinical methods in coordinated proactive monitoring programs to detect the emergence and deter the spread of resistance to ACTs.

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