Chloroquine clinical failures in P. falciparum malaria are associated with mutant Pfmdr-1, not Pfcrt in Madagascar.

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Chloroquine Clinical Failures in *P. falciparum* Malaria Are Associated with Mutant *Pfmdr-1*, Not *Pfcrt* in Madagascar

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Abstract

Molecular studies have demonstrated that mutations in the *Plasmodium falciparum* chloroquine resistance transporter gene (*Pfcr*) play a major role in chloroquine resistance, while mutations in *P. falciparum* multidrug resistance gene (*Pfmdr-1*) act as modulator. In Madagascar, the high rate of chloroquine treatment failure (44%) appears disconnected from the overall level of *in vitro* CQ susceptibility (prevalence of CQ-resistant parasites <5%) or *Pfcr* mutant isolates (<1%), strongly contrasting with sub-Saharan African countries. Previous studies showed a high frequency of *Pfmdr-1* mutant parasites (>60%) of isolates, but did not explore their association with *P. falciparum* chloroquine resistance. To document the association of *Pfmdr-1* alleles with chloroquine resistance in Madagascar, 249 *P. falciparum* samples collected from patients enrolled in a chloroquine *in vivo* efficacy study were genotyped in *Pfcr/Pfmdr-1* genes as well as the estimation of the *Pfmdr-1* copy number. Except 2 isolates, all samples displayed a wild-type *Pfcr* allele without *Pfmdr-1* amplification. Chloroquine treatment failures were significantly associated with *Pfmdr-1* 86Y mutant codon (OR = 4.6). The cumulative incidence of recurrence of patients carrying the *Pfmdr-1* 86Y mutation at day 0 (21 days) was shorter than patients carrying *Pfmdr-1* 86N wild type codon (28 days). In an independent set of 90 selected isolates, *in vitro* susceptibility to chloroquine was not associated with *Pfmdr-1* polymorphisms. Analysis of two microsatellites flanking *Pfmdr-1* allele showed that mutations occurred on multiple genetic backgrounds. In Madagascar, *Pfmdr-1* polymorphism is associated with late chloroquine clinical failures and unrelated with *in vitro* susceptibility or *Pfcr* genotype. These results highlight the limits of the current *in vitro* tests routinely used to monitor CQ drug resistance in this unique context. Gaining insight about the mechanisms that regulate polymorphism in *Pfmdr1* remains important, particularly regarding the evolution and spread of *Pfmdr-1* alleles in *P. falciparum* populations under changing drug pressure which may have important consequences in terms of antimarial use management.

Introduction

*Plasmodium falciparum* resistance to chloroquine (CQ) has emerged at least from six independent foci (South East Asia, Venezuela, Colombia, Papua New Guinea, India and Philippines) in the late 1950s and in the 1960s [1,2,3]. Molecular evolutionary studies have demonstrated that *P. falciparum* CQ-resistant parasites from South-East Asia have entered in East Africa (Kenya and Tanzania) in the late 1970s and spread across the African continent within two decades [2]. *P. falciparum*-related deaths rose sharply after the spread of CQ-resistant parasites in sub-Saharan Africa, affecting mostly children under 5 years of age [4,5,6].

CQ-resistance in *P. falciparum* shows some biological similarities with the multiple drug resistance phenotype of mammalian tumour cells, as both involve expulsion of drug out of the cytosol of the cell and can be reversed by calcium channel antagonists such as verapamil [7]. Based on molecular allele exchange studies and analysis of genetic crosses, it is today generally accepted that the major role in CQ resistance is determined by polymorphisms in *Pfcr*, a gene encoding a transporter which promotes, in its mutated forms, drug efflux from the parasite digestive vacuole, while *Pfmdr-1* modulates the level of *in vitro* CQ-resistance [3,8,9]. In addition, mutations or amplifications of *Pfmdr-1* gene can play a significant role in *P. falciparum* resistance to diverse antimalarials such as mefloquine, quinine or artemisinin derivatives [10]. Specific combinations of *Pfcr* and *Pfmdr-1* alleles, resulting in varying responses to CQ (and amodiaquine), appeared geographically restricted, which may explain why some field studies


Editor: Georges Snounou, Université Pierre et Marie Curie, France

Received June 22, 2010; Accepted September 12, 2010; Published October 13, 2010

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Funding: This study was supported by grants from the Institut de Médecine et d’Épidemiologie Appliquée (IMEA), Fondation Léon MBA, Paris, France and the Genomics in Medicines, Pasteur Genopole, Pasteur Institute, France. Samples collection in Madagascar was funded by the Global Fund to Fight AIDS, Tuberculosis and Malaria, round 3 (Community Action to Roll Back Malaria, grant no. MDG-304-G05-M). Valerie Andriantsoanirina is a graduate PhD student funded by the Institut Pasteur de Madagascar (Bourse “Girard”) and the Direction des Affaires Internationales (Institut Pasteur). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

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reported an association between \textit{Pfmdr-1} polymorphisms and CQ resistance and other studies did not [9].

In Madagascar, CQ was first introduced in 1945 and was largely used during more than 50 years, in particular in a campaign for malaria prevention treatment in children [11]. A shortage in CQ supply in the 1970s was followed by a large-scale epidemic in the late 1980s. Wide-scale access to CQ treatment resumed afterwards, and CQ is still used today though its replacement by the artesunate plus amodiaquine combination therapy is recommended as first-line treatment for uncomplicated cases since 2005. The first clinical cases of CQ resistance were suspected in 1975. In 1981, other cases of treatment failures were reported and in \textit{vitro} tests showed isolates with high 50\% inhibitory concentration for CQ (IC$_{50}$) indicative of \textit{in vitro} CQ-resistance. However, during the 1981-2008 period, several studies outlined lower rates of \textit{in vivo} CQ-resistance and therapeutic failures in Madagascar than in other African countries [11]. Currently, the prevalence of \textit{in vitro} CQ resistance does not exceed 5\% of isolates and \textit{Pfcr} mutant parasites are found in less than 1\% of collected isolates [12]. Surprisingly, these figures appear disconnected from the high level of clinical treatment failures (44\%) most of which (\(\sim\)90\%) are late clinical/parasitological treatment failures. Interestingly, previous surveys outlined the presence of \textit{Pfmdr-1} mutations in more than 60\% of isolates, suggesting that \textit{Pfmdr-1} mutants could be responsible for CQ clinical failure in Madagascar.

The aim of the present work was to document the association of \textit{Pfmdr-1} with \textit{in vivo} CQ clinical failure and \textit{in vitro} CQ resistance. To this end, two independent sets of \textit{P. falciparum} samples collected in 2006-2007 from \textit{in vivo} and \textit{in vitro} susceptibility studies were genotyped for \textit{Pfcr} and \textit{Pfmdr1} genes as well as the estimation of the \textit{Pfmdr-1} copy number. Isolates that meet the inclusion criteria were included in the association analysis (240 from \textit{in vivo} study and 90 from \textit{in vitro} testing). In addition, the evolutionary dynamics of the \textit{Pfmdr-1} locus in the Malagasy parasite populations were also assessed. Our data show that in this particular setting, \textit{Pfmdr-1} polymorphism on multiple genetic backgrounds, in absence of mutations in \textit{Pfcr} gene or \textit{Pfmdr-1} gene amplification was significantly associated with late chloroquine clinical failures and unrelated with the overall level of \textit{in vitro} CQ susceptibility, raising the limits of the current \textit{in vitro} tests routinely used to monitor CQ drug resistance.

\section*{Materials and Methods}

\subsection*{Study Sites}

\textit{P. falciparum} samples from eight sentinel sites involved in the monitoring of the antimalarial drug resistance in Madagascar [12] were studied. The collection sites were located in the four main malarious epidemiological strata: Ejeda and Ihosy in the South (sub-desert stratum, epidemic prone), Maevatana and Miandrivazo in the West (tropical stratum, seasonal and endemic area), Tsiraromandidy and Moramanga in the foothills of the Central Highlands (highlands stratum, low-endemic area) and Farafangana and Andapa in the East (equatorial stratum, perennial endemic area). Malaria transmission, estimated by annual entomological inoculation rate (EIR), number of bites of infected anophelines per person sleeping indoors), varied according to the sites from 0.2 (in the South) to 240 (in the East) [14]. The prevalence of anti-\textit{MSP1} antibodies used as proxy of the burden of malaria in each site was previously determined at the same collection period [13] and were included in our analysis.

\subsection*{Patients and \textit{in vivo} study}

In 2006-2007, multi-site prospective chemotherapeutic studies were carried out for assessing the therapeutic efficacy of antimalarial therapies recommended by the National Malaria Control Programme (NMCP) (registration number ISRCTN-95617335) [13]. Patients with microscopically confirmed uncomplicated \textit{P. falciparum} malaria were randomized into one of the four treatment groups: Chloroquine (CQ), Amodiaquine (AQ), Sulfa-doxine-Pyrimethamine association (SP) and Artesunate-Amodiaquine combination (ASAQ). According to the 2003 WHO protocol, patients aged from six months to 15 years, presenting with \textit{P. falciparum} mono-infection (parasitaemia from 1,000 to 200,000/\(\mu\)L), axillary temperature \(\geq\)37.5\(^\circ\)C, body weight >5 kg, haemoglobin (Hb) \(\geq\)5 g/dL, without severe malnutrition, signs of severity, or a concomitant disease were included in the clinical trial. Once written informed consent was given by the patient or the guardian and after randomization, patients were administered either CQ (10 mg/kg on days 0 and 1, and 5 mg/kg on day 2), AQ (10 mg/kg on days 0, 1, and 2), SP (25 mg/kg sulfadoxine and 1.25 mg/kg pyrimethamine as a single dose on day 0) or ASAQ (AS: 4 mg/kg on days 0, 1, and 2 and AQ:10 mg/kg on days 0, 1, and 2) and seen on days 1, 2, 3, 7, 14, 21 and 28, or any intervening day if they were unwell for malaria infection to assess the clinical and parasitological efficacy of the drug regimen.

\section*{Collection of \textit{P. falciparum} isolates for \textit{in vitro} drug sensitivity testing}

As part of the surveillance of antimalarial drug resistance in Madagascar, fresh \textit{P. falciparum} clinical isolates were routinely collected in 2006-2007 from symptomatic patients prior to treatment in six of the eight sites (Ihosy, Maevatana, Miandrivazo, Tsiraromandidy, Moramanga and Farafangana). Venous blood samples were collected in tubes coated with EDTA (Vacutainer tubes, Becton Dickinson, Rutherford, NJ, USA), from malaria-positive patients (>2 years old) after they gave their consent for participation in the study (approval number 007/ SANFP/2007). Presence of malaria parasites was evaluated by using a rapid diagnostic test based on the detection of \textit{Plasmodium}-specific lactate dehydrogenase (pLDH) (OptiMAL-IT, DiaMed AG, Cressier sur Morat, Switzerland). Positive patients were treated with the artesunate-amodiaquine combination, according to the National Malaria Control Programme (NMCP), and samples were sent to the Malaria Research Unit of the Institut Pasteur of Madagascar in a controlled cool box at 4\(^\circ\)C. Drug sensitivity assays were done using the classical isotopic 48-h test, as described [12], on blood samples with parasitaemia \(\geq\)0.1\%, available within 48 h after blood collection and from patients declaring no antimalarial drug intake during the previous 7 days. For \textit{CQ} sensitivity assay, three control wells without drug were used as control and each concentration ranging from 12.5 to 1,600 nM was studied in duplicate or triplicate. The IC$_{50}$, i.e. the drug concentration corresponding to 50\% of \textit{H}-hypoxanthine uptake by the parasites in drug-free control wells, was determined by probit/logit regression analysis. The quality of the assays was controlled by using \textit{P. falciparum} reference lines (3D7 Africa, \textit{CQ}-sensitive clone and FCML29 Cameroon, \textit{CQ}-resistant clone).

\section*{DNA extraction}

Parasite DNA was available from whole blood or capillary blood transferred to filter paper (Whatman, Maidstone, UK). DNA was extracted from blood spots with Instagene\textsuperscript{\textregistered} Matrix resin
CQ Resistance in Madagascar

was used as the quality control in each run. Reference DNA clone W2 (which has three copies of the ß-tubulin (used as a housekeeping gene) was measured relative to the numbers in two standard calibrator L reaction mixtures. For each run, the 
Pfmdr-1 copy number determination

The 
Pfmdr-1 allele; ninety of them, meeting criteria of inclusion for the analysis were (i) patients with complete 28-days follow-up, (ii) absence of reinfection, (iii) wild-type 
Pfcr alleles and (iii) successful determination of 
Pfmdr-1 alleles. The Mann-Whitney U test or Kruskal-Wallis method were used for non-parametric comparisons, and Student’s t test or one-way analysis of variance for parametric comparisons. For categorical variables, Chi-squared or Fisher’s exact tests were used to assess significant differences in proportions. Odds ratios (OR) and their 95% confidence intervals (95%CI), describing the association between CQ clinical outcomes and exposure variables were determined by conditional logistic regression.

For the multivariate analysis, variables with P-values <0.25 were initially introduced into the model and removed following a backwards-stepwise selection procedure to leave only those with a P-value <0.05 in the final model. The relation between 
Pfmdr-1 86Y mutation in isolates from day of recurrence and clinical response to CQ treatment was assessed by survival analysis using the Kaplan-Meier method and the log-rank test. The median asexual parasite densities clearance time (in percent of value on day 0) was also compared between patients according to the 
Pfmdr-1 alleles (86N and 86Y) found at day 0 (and day of recurrence). All reported P-values are two-sided and were considered statistically significant if less than 0.05.

Genetic diversity was assessed by Nei’s unbiased expected heterozygosity (He) from haplotype data and calculated as 

Analysis microsatellite flanking 
Pfmdr-1

Two microsatellite markers (MS loci 956456 and 957861), located on chromosome 5 and extending ~10 kb downstream the 
Pfmdr-1 gene were used to determine the evolutionary history of 
Pfmdr-1 alleles. Microsatellite polymorphism was analyzed using a nested PCR strategy, as previously described by Mehlotra et al. [9]. Microsatellite PCR products were genotyped on the basis of size, using a GeneScan 500 LIZ size standard on an ABI Prism 3730 XL DNA analyzer.

Assessment of isolate clonality

The number of genotypes present in isolates collected from in vivo and in vitro assays, was estimated by using an allelic family-specific nested PCR (MAD20, K1, and RO33 for 
Pfmsp-1 and 3D7 Africa and FC27 for 
Pfmsp-2) [12]. Clonality was defined as the highest number of alleles detected at either of the two loci and used to classify isolates as monoclonal or polyclonal and to distinguish recrudescence from new infection for all patients failing therapy after day 7 (isolates from day 0 and day of recurrence). All PCR amplifications contained a positive control (genomic DNA from strains W2, HB3, and 3D7 Africa) and a negative control (no target DNA).

Statistical analysis

Data were entered and verified using Microsoft Excel® software, and analyzed using EpInfo 6.04® software (Centers for Disease Control and Prevention, Atlanta, GA, United States) and XLSTAT® for Windows XP (Addinsoft, Paris, France). For in vivo clinical trial samples, criteria of inclusion for the analysis were (i) patients with complete 28-days follow-up, (ii) absence of reinfection, (iii) wild-type 
Pfcr alleles in isolates collected at day 0 and day of recurrence and (iv) successful determination of 
Pfmdr-1 alleles in isolates from day 0 and day of recurrence. For in vitro testing samples, criteria of inclusion for the analysis were (i) monoclonal isolates by using 
Pfmsp-1/Pfmsp-2 genotyping, (ii) wild-type 
Pfcr alleles and (iii) successful determination of 
Pfmdr-1 alleles.

The study protocol was reviewed and approved by the Ethics Committee of the Ministry of Health of Madagascar (approval number 007/SANPF/2007; registration number ISRCTN-36517335). Informed written consent was provided by all patients or their parents/guardians before inclusion in the study.

Results

In vivo and in vitro sample collection

In the in vivo clinical trial, a total of 8363 febrile children attending in health centres from the sentinel sites were screened for 
P. falciparum malaria and 1873 were microscopically positive for 
P. falciparum. They were assigned in different treatment groups. Among the 320 patients treated with CQ, 240 of them (75%) met the study analysis criteria. According to the WHO 2003 protocol, 119 patients were classified as cured and 121 as failing treatment (13 patients failed before day 7, 20 patients between days 8–14, 46 patients between days 15–21 and 42 patients between days 22–28). Late treatment failures accounted for 89.3% of the overall treatment failures. Details are given in figure 1.

Among the 420 
P. falciparum isolates tested for in vitro susceptibility to CQ, 372 (88.6%) were successfully assayed. All isolates presented a wild type 
Pfcr allele; ninety of them, meeting
the study analysis criteria, were included in the analysis. Among the 90 isolates in vitro tested, the geometric mean and the median of CQ IC50 was 18.7 nM (95%CI 14.7–23.7 nM) and 22 nM (95%CI 16.2–25.0 nM), respectively.

**Pfmdr-1 alleles, copy number of Pfmdr-1 gene**

Pfmdr-1 alleles were determined for the 240 isolates from the in vivo efficacy study (day 0 and day of recurrence, when available) and for 90 isolates for which the IC50 for CQ was determined. Amongst in vivo isolates, three of five major SNPs previously related to CQ-resistance were observed at day 0, N86Y (147/240, 61.2%), Y184F (172/240, 71.7%), and D1246Y (80/240, 33.3%), present in eight different alleles: the wild-type allele NYD (11.2%), three single-mutant alleles (NYD, 22.9%; YYD, 14.6% and NYY, 1.2%), three double-mutant alleles (YFD, 7.9%; NFD, 3.3% and YFD, 1.2%) and one triple-mutant allele (YFY, 27.5%). Discordant alleles were found in 24 isolates between isolate from day 0 (wild-type NYD) and isolate from day of recurrence (13 single-mutant alleles YYD, 8 single-mutant alleles NFD and 3 double-mutant alleles YFD). There was a non-random association between the N86Y and D1246Y loci ($R^2 = 0.23$, $P = 0.004$) and F184Y and D1246Y loci ($R^2 = 0.28$, $P = 0.0003$).

Amongst in vitro isolates, only three isolates had an IC50>100 nM: one triple-mutant allele (YFY, IC50 = 142 nM), one double-mutant allele (YFD, IC50 = 126 nM) and one single-mutant allele (NFD, IC50 = 140 nM). Comparison of geometric mean IC50 between isolates harbouring different Pfmdr-1 alleles showed no significant difference: 18.0 nM ($n = 37$, 95%CI 12.1–26.6 nM) in Pfmdr-1 86N isolates compared to 19.2 nM ($n = 53$, 95%CI 14.1–26.0 nM) in Pfmdr-1 86Y isolates, 14.3 nM ($n = 72$, 95%CI 8.2–25.0 nM) in Pfmdr-1 184Y isolates compared to 20.0 nM ($n = 18$, 95%CI 15.3–26.1 nM) in Pfmdr-1 184F isolates and 21.4 nM ($n = 61$, 95%CI 13.5–36.7 nM) in Pfmdr-1 1246D isolates compared to 18.6 nM ($n = 21$, 95%CI 12.3–28.3 nM) in Pfmdr-1 1246Y isolates.

The Pfmdr-1 copy number was determined for 290 isolates (219 from in vivo studies and 71 from in vitro assays). The mean copy number was 0.93 (ranging from 0.74 to 1.33). When the value was rounded to the nearest integer, no Pfmdr-1 amplification was observed.

Figure 1. Flowchart of patients. Flowchart of patients: Enrollment, Randomization, Follow-up, Molecular genotyping.
doi:10.1371/journal.pone.0013281.g001
**Pfmdr-1 microsatellite polymorphism**

The polymorphisms in two microsatellite loci flanking the wild-type coding sequence and mutant-type alleles (single-mutants to triple-mutant) are shown in Figure 2. The MS locus 956456 had 6 alleles (ranging from 210 to 228 bp) and the MS locus 957861 had 4 alleles (ranging from 179 to 185 bp), displaying 7 different haplotypes. Microsatellite markers were moderately polymorphic for wild- and mutant-type alleles with a mean Nei’s unbiased expected heterozygosity (He) ranging from 0.26 (NYD) to 0.66 (NYF). The Wright’s fixation index analysis showed a significant absence of genetic differentiation between allele populations.

**In vivo outcomes and risk factors associated to CQ treatment failure**

To test whether any collected variables (ecological environment, patients and isolates characteristics) were associated with clinical response to CQ treatment, we compared them between both cured- and failing treatment patient groups (Table 1). Among these, four variables were significantly associated with the risk of CQ treatment failure: (i) prevalence of anti-PfMSP-1 antibodies previously estimated in the site (P<0.04, 1.8-fold increased risk of CQ treatment failures in area of unstable malaria), (ii) age group (P<0.0001), increased risk of CQ clinical failures inversely correlated to age: 1.6-fold in the 6–10 years age group and 4.5-fold in the 0.5–5 years age group, (iii) haemoglobin concentration at day 0 (P<0.0002, 2.4-fold increased risk of CQ treatment failures for people with haemoglobin concentration <10 g/dl at day 0) and (iv) presence of the Pfmdr-1 86Y mutation in isolates collected on day 0 (P=0.009, 2-fold increased risk of CQ treatment failures) or on day of recurrence (P<0.0001, 4.2-fold increased risk of CQ treatment failures).

In the multivariate analysis, variables with P-values <0.25 were initially introduced into the model (prevalence of anti-PfMSP-1 antibodies, gender, age, haemoglobin concentration at day 0, multiplicity of infection, presence of Pfmdr-1 codon mutation at position 86 in isolates from day 0 and day of recurrence and at position 184 in isolates from day of recurrence). Following a backwards-stepwise selection procedure, CQ treatment failures were significantly associated with presence of Pfmdr-1 codon mutation at position 86 in the parasites collected on day of recrudescence (OR = 4.6, 95%CI 2.3 to 8.9, P<0.0001), age (OR = 1.2, 95%CI 1.1 to 1.3, P = 0.0002) and prevalence of antiPfMSP-1 antibodies (OR = 1.02, 95%CI 1.0 to 1.05, P = 0.02).

Asexual parasite clearance following CQ-treatment displayed significant differences according to Pfmdr-1 allelic form at position 86 at day 1 (Pfmdr-1 86N: 77.1%, n = 89 and Pfmdr-1 86Y: 95.3%, n = 151, P = 0.0001), day 7 (Pfmdr-1 86N: 3.7%, n = 85 and Pfmdr-1 86Y: 34.3%, n = 142, P<0.0001) and day 14 (Pfmdr-1 86N: 2.0%, n = 78 and Pfmdr-1 86Y: 54.9%, n = 129, P<0.0001) (Figure 3).

**Cumulative incidence of recurrence of patients and Pfmdr-1 86 mutation**

The relation between Pfmdr-1 86Y mutation in isolates from day 0 (or day of recrudescence, if available) and clinical response to CQ treatment by using the time of recurrence, showed a markedly difference between the two curves (Figure 4). The cumulative incidence of recurrence of patients carrying the Pfmdr-1 86Y allele (n = 163, median time of recurrence 21 days) was significantly shorter than patients carrying Pfmdr-1 86N (n = 77, median time of recurrence 28 days). The log-rank test shows that the two curves differ significantly (P<0.0001) and the Pfmdr-1 N86Y allele had a significant influence on the time of recurrence. The hazard ratio comparing the hazards in the two groups was estimated at 0.36 (95%IC: 0.23–0.55).

**Discussion**

In the isolates from Madagascar studied here which harboured a wild type Pfcr, Pfmdr-1 polymorphism, in the absence of any noticeable gene amplification, seems to play a major role in late chloroquine clinical failures without affecting the overall level of in vitro CQ susceptibility. This situation contrasts with findings from multiple settings across the African continent, where CQ resistance depends primarily on mutations in Pfcr gene and on additional mutations in Pfmdr-1 gene, which may increase the level of resistance afforded by Pfcr. Pfcr mutant alleles seem universally found, albeit with different haplotypes in different geographical regions [22]. The association of Pfmdr-1 with CQ resistance was not found in some areas [10], possibly reflecting the fact that the impact of the Pfcr/Pfmdr-1 combination mutation depends on the genetic background of the strain [23,24] as demonstrated by studies with genetically manipulated lines or recombinant progeny of experimental crosses [23,24] and the history of use of the antimalarial drugs (chloroquine and quinine being the two maims drugs used in Madagascar).

The observation that parasites with a wild Pfcr allele were associated with a high rate of therapeutic failures of chloroquine is totally new. Several hypothesis may account for these findings. First, a particular Pfcr haplotype, restricted to the Malagasy area and different from previously described resistant haplotypes, was present but not detected during this study. As we sequenced for all isolates a large part of Pfcr gene including five codons consistently associated with CQ resistance in other settings, this hypothesis appears unlikely. It also is worth noting that so far the rare Pfcr mutant alleles found in two sites from Madagascar (Andapa and Tsiraimomandidy) had a CVIET or CVIET haplotype [25] and most likely were imported from the neighbouring Comoros Islands. Why did the CVIET haplotype not spread largely across Madagascar in spite of a significant drug pressure, as it did in Asia and Africa and even in the neighbouring Comoros Islands, remains to explain. A second hypothesis is that another gene, distinct from Pfcr and not identified at present, is the main responsible for CQ resistance in Madagascar. Though this hypothesis cannot be formally ruled out, it appears also unlikely in view of the observed, strong association with Pfmdr1. A third possibility is, that the Pfcr allele is present at a low fraction in the time 0 sample, too low to be detected (masked by the wild type allele and not detected by the PCR followed by DNA sequencing methodology) and too low to translate into a shifted IC50 in vitro (as these parasites represent a low fraction, they incorporate a low amount of 3H hypoxanthine and remain unnoticed). We also think this possibility unlikely, as no mutant Pfcr could be detected in the samples collected from patients with late therapeutic failures. Indeed, the seminal studies by Djimde et al. in Mali showed that the minority CQ resistant clone selected during a treatment and causing a therapeutic failure accounted for a substantial proportion of the parasites on the day of recurrence [26]. Juliano et al. [27], using a nonradioactive heteroduplex tracking assay, reported recently that in a unique series of 17 patients in Madagascar, two (11.7%) harboured Pfcr CVIET-resistant haplotype. The proportion of mutant parasites was 1.7 and 2.9% of the total parasite recurrent population obtained 14 days after the onset of CQ treatment, and neither of the two patients harboured any detectable CVIET parasites prior to treatment. In both cases the “recurrent” isolate 14 days after treatment by CQ contained a vast majority of CQ susceptible...
Figure 2. Prevalence of the two microsatellite loci flanking Pfmdr-1 gene. Distribution and prevalence of the two microsatellite loci flanking Pfmdr-1 gene (MS 956456, panel A and MS 957861, panel B) in 53 Plasmodium falciparum isolates collected from Madagascar in 2006–2007. doi:10.1371/journal.pone.0013281.g002

Amino-acids, in single letter code, conferring resistance are shown in bold and underlined. The amino-acid residues at positions 86, 184 and 1246 are indicated.
We did not detect any genetic loci other than parasites (98.3% and 97.1% respectively), indicating that parasite genetic loci other than Pfert came into play in these recurrences. We did not detect any Pfert resistant haplotype on the recurrence day of the 121 therapeutic failures of our series. Whether this reflects a somewhat lower sensitivity of our methodology compared to Juliano et al remains to be determined, but it is certainly consistent with the conclusion that these recurrences are not associated with selection of a minority, mutant Pfert clone. The fourth hypothesis is that mutant alleles of Pfmdr-1 play a major role in CQ clinical outcome. Ten years ago, transfection methods were used to explore the role of Pfmdr-1 mutations in CQ resistance [28]. Introduction of wild-type polymorphisms into the resistant 7G8 line resulted in reduction of CQ resistance but introduction of mutations in the susceptible D10 line did not confer CQ resistance, which suggested that Pfmdr-1 was not sufficient by itself to confer resistance. Lessons drawn from a recent genetic cross however indicate that observations with one or the other P. falciparum line are not directly applicable to all lines [24]. Different phenotypes of CQ resistance (and amodiaquine resistance) exist in the world, differing in different geographical locations.

Table 1. Univariate (conditional logistic regression) analysis of risks factors associated to CQ-treatment failure (recrudescence), Madagascar, 2006–2007.

<table>
<thead>
<tr>
<th>Exposure variables</th>
<th>Treatment outcome</th>
<th>P-value</th>
<th>OR^a</th>
<th>95% CI^b</th>
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<tr>
<td><strong>Collection sites</strong></td>
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<tr>
<td>Epidemiological strata^1</td>
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<td>Failed</td>
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<td></td>
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<td>32</td>
<td>52.5%</td>
<td>1.1</td>
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<td>Sub-desert</td>
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<td>50.0%</td>
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<td>EIR (number of bites of infected anophelines per person sleeping indoors)^2</td>
<td>&lt;5 (reference)</td>
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<td>Failed</td>
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</tr>
<tr>
<td></td>
<td>&gt; = 5</td>
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<td>49.5%</td>
<td>0.95</td>
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<tr>
<td>Prevalence of anti-PfMSP-1 antibodies^3</td>
<td>&lt;40% (reference)</td>
<td>n</td>
<td>Failed</td>
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<tr>
<td></td>
<td>&gt; = 40%</td>
<td>72</td>
<td>56.7%</td>
<td>0.57</td>
</tr>
<tr>
<td><strong>Patient characteristics</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male gender</td>
<td>121</td>
<td>44.3%</td>
<td>0.2^8</td>
<td>0.78</td>
</tr>
<tr>
<td>Age</td>
<td>mean (± SD), year</td>
<td>121</td>
<td>48 (3.7)</td>
<td>&lt;0.0001^7</td>
</tr>
<tr>
<td></td>
<td>0.5–5 (reference)</td>
<td>79</td>
<td>64.8%</td>
<td>35.2%</td>
</tr>
<tr>
<td></td>
<td>6–10</td>
<td>33</td>
<td>39.5%</td>
<td>60.5%</td>
</tr>
<tr>
<td></td>
<td>11–15</td>
<td>11</td>
<td>28.9%</td>
<td>71.1%</td>
</tr>
<tr>
<td>Temperature (°C) at day 0. mean (± SD)</td>
<td>121</td>
<td>38.4 (0.7)</td>
<td>0.61^1</td>
<td>1.0824</td>
</tr>
<tr>
<td>Haemoglobin concentration (g/dL) at Day 0. mean (± SD)</td>
<td>121</td>
<td>9.4 (2.4)</td>
<td>0.0002^7</td>
<td>0.8</td>
</tr>
<tr>
<td>Previous CQ in-take (%)</td>
<td>121</td>
<td>82</td>
<td>0.57*</td>
<td>0.97</td>
</tr>
<tr>
<td><strong>Clinical isolates</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Parasitaemia density at Day 0 (no.of parasites/µL). mean (± SD)</td>
<td>121</td>
<td>41,435 (60,928)</td>
<td>0.8^1</td>
<td>1</td>
</tr>
<tr>
<td>Multiplicity of infection (MOI) mean. (± SD)</td>
<td>121</td>
<td>1.9 (0.9)</td>
<td>0.06^1</td>
<td>0.39</td>
</tr>
<tr>
<td>Pfmdr-1 86Y,% at Day 0</td>
<td>121</td>
<td>67.7%</td>
<td>0.009^3</td>
<td>2.01</td>
</tr>
<tr>
<td></td>
<td>at Day of recrudescence</td>
<td>121</td>
<td>82.6%</td>
<td>&lt;0.0001^4</td>
</tr>
<tr>
<td>Pfmdr-1 184F,% at Day 0</td>
<td>121</td>
<td>71.9%</td>
<td>0.90^1</td>
<td>1.02</td>
</tr>
<tr>
<td></td>
<td>at Day of recrudescence</td>
<td>121</td>
<td>81.0%</td>
<td>0.08^1</td>
</tr>
<tr>
<td>Pfmdr-1 1246Y. % at Day 0</td>
<td>121</td>
<td>31.3%</td>
<td>0.98^1</td>
<td>0.99</td>
</tr>
</tbody>
</table>

^1Treatment failure was based on the World Health Organization 28-day drug efficacy test and monitoring and corrected by PCR genotyping as described in Materials and Method section;
^2Chi-squared test;
^3Fisher exact test;
^4ANOVA test (or if P-value of the Bartlett’s test for inequality of population variances was <0.05, Mann–Whitney/Wilcoxon two-sample tests);^5OR: odds ratio;
^695% Confidence Interval;
^7Discordant alleles found in 16 isolates between day 0 (wild-type NYD) and day of recrudescence (13 single-mutants YFD and 3 double-mutants YFD);
^8Discordant alleles found in 8 additional isolates between day 0 (wild-type NYD) and day of recrudescence (8 single-mutants NFD);
^9According to [18];^10According to [19] and ^11According to [20].

References: [18, 19, 20]
Figure 3. Parasites curves following CQ-treatment according Pfmdr-1 alleles. Asexual parasites curves following CQ-treatment according Pfmdr-1 mutation at position 86 in isolates from patients included in the analysis, Madagascar in 2006–2007.

doi:10.1371/journal.pone.0013281.g003

Figure 4. Curves of cumulative incidence of recurrence of patients over the 28-day follow-up period. Kaplan-Meier curves of cumulative incidence of recurrence of patients over the 28-day follow-up period according Pfmdr-1 mutation at position 86 in isolates of day of recurrence from patients included in the analysis, Madagascar in 2006–2007.

doi:10.1371/journal.pone.0013281.g004
areas. The contribution of Pfmdr-1 mutations in CQ resistance varies depending on the particular Pfcr haplotype with which they are associated [24].

The situation with regard to CQ resistance in Madagascar is unique. There were high rates of therapeutic failures, but those failures occurred in close to 90% of cases more than 7 days after treatment (Late Treatment Failures, LTFs) whereas in sub-Saharan Africa the proportion of LTFs is usually near 20% [29,30,31,32,33]. The proportion of isolates with detectable Pfcr mutant parasites was totally disconnected from the rate of clinical failures. Moreover, no selection of Pfcr mutant haplotypes between the first day of treatment and the day of recurrence was observed, which contrasted strongly with studies conducted in sub-Saharan countries [26]. We found also a selection of the N96Y or Y184F mutant alleles between the first day of treatment and the day of recurrence (10%), suggesting that Pfmdr-1 has a major role in CQ clinical failures in Madagascar. However, this particular type of resistance was not reliably detected by the classical isotopic 48-h test used in our study, as only 3.3% of isolates showed phenotypic resistance (IC₅₀>100 nM). The follow-up of circulating parasite densities over time in recurrent malaria episodes, showing rapid (but not complete) asexual parasites clearance within 72-hours following first day of treatment (Figure 3), was consistent with the apparent in vitro susceptibility of the isolates obtained in therapeutic failure cases. These results highlight the limits of the in vitro tests routinely used to monitor the antimalarial drug resistance. Based on the detection of the “trophozoites to schizonts” growth during a single parasite erythrocytic cycle, it appears that such in vitro assays can accurately detect high levels of resistance (corresponding usually to early treatment failures) but are unable to evaluate phenotypic resistance yielded by slow acting molecular mechanisms (or unusual mechanism), likely related to late failure treatments [23,34].

The analysis of microsatellites flanking Pfmdr-1 showed that mutations occurred on multiple genetic backgrounds (Figure 2). As previously described by Mehlotra et al. [9], we did not observe a strong reduction of heterozygosity in the CQ resistant parasite population, underlining that drug pressure from CQ treatment exerts a weaker selection on Pfmdr-1 than on Pfcr. This “soft” selection of Pfmdr-1 mutant alleles may also be due to quinine pressure, as quinine was for a long time until recently the antimalarial most commonly prescribed by practitioners in Madagascar [12]. The spread of Pfmdr-1 mutant alleles may reflect a better fitness of parasites harbouring them or a better efficacy of the transmission [33].

Apart from the Pfmdr-N96Y allele, the main risk factors associated with CQ therapeutic failures, lower age and lower prevalence of anti-PfMSP-1 antibodies, were inversely correlated with the immune status of patients. These results are similar with what has been reported for Pfcr mutants in Mali and other countries where increasing age, reflecting the progressive acquisition of partial immunity, helped in the clearance of resistant parasites [26]. Hence, the immune status of patients probably explains why a significant proportion of patients having isolates harbouring a mutant Pfmdr-1 allele were successfully cured by CQ in our series.

At present, CQ is being replaced by newer artemisinin-based combination drugs, such as artesunate-amodiaquine as first-line treatment in uncomplicated falciparum malaria in Madagascar. As Pfmdr-1 is a major modulator of resistance to these drugs [10], monitoring in the future in which measure the high proportion of Pfmdr-1 mutants observed in Madagascar can have an impact on the resistance of these antimarialarias, even if at the moment this impact seems modest [11]. More generally, gaining insight about the mechanisms that regulate the genetic variation at Pfmdr1 is important, particularly regarding the evolution and spread of Pfmdr-1 alleles in P. falciparum populations under changing drug pressure which may have important consequences in terms of antimalarial use management.

**Acknowledgments**

We thank the patients and healthcare workers involved in the national network for the surveillance of malaria resistance in Madagascar (Réseau d’Etude de la Résistance, RER) from which these samples were obtained, and the staff of the Ministry of Health of Madagascar for their collaboration.

**Author Contributions**

Conceived and designed the experiments: AR OMP RD DM. Performed the experiments: VA MT MJ SR RR. Analyzed the data: VA CB OMP RD. Contributed reagents/materials/analysis tools: AR CB OMP DM. Wrote the paper: VA OMP RD DM.