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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 antimalarial use management.

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**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
reported an association between 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 vitro tests showed isolates with high 50% inhibitory concentration for CQ (IC50) indicative of in vitro CQ-resistance. However, during the 1981-2008 period, several studies outlined lower rates of in vitro CQ-resistance and therapeutic failures in Madagascar than in other African countries [11].

Currently, the prevalence of in vitro CQ resistance does not exceed 5% of isolates and Pfert 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%) [13], most of which (~90%) are late clinical/parasitological treatment failures. Interestingly, previous surveys outlined the presence of Pfmdr-1 mutations in more than 60% of isolates, suggesting that Pfmdr-1 mutants could be responsible for CQ clinical failure in Madagascar.

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

Materials and Methods

Study Sites

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), Tsiroanomandidy 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-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.

Patients and 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-95317335) [13]. Patients with microscopically confirmed uncomplicated P. falciparum malaria were randomized into one of the four treatment groups: Chloroquine (CQ), Amodiaquine (AQ), Sulpha-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 P. falciparum mono-infection (parasitaemia from 1,000 to 200,000/µL), axillary temperature ≥37.5°C, body weight >5 kg, haemoglobin (Hb) ≥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 regimens.

Collection of P. falciparum isolates for in vitro drug sensitivity testing

As part of the surveillance of antimalarial drug resistance in Madagascar, fresh 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, Tsiroanomandidy, 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/ SANPF/2007). Presence of malaria parasites was evaluated by using a rapid diagnostic test based on the detection of 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°C. Drug sensitivity assays were done using the classical isotopic 48-h test, as described [12], on blood samples with parasitaemia ≥0.1%, available within 48 h after blood collection and from patients declaring no antimalarial drug intake during the previous 7 days. For CQ sensitivity assay, three control wells without drug were available within 48 h after blood collection and from patients controlled by using reference lines (3D7 Africa, CQ-sensitive clone and FCML29 Cameroon, CQ-resistant clone).

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® Matrix resin
(BioRad®, Marnes la Coquette, France), according to the manufacturer’s instructions or directly from 100 μl of whole blood by using the phenol-chloroform method [16]. Parasite species was confirmed by using real-time polymerase chain reaction as described by de Monbrison et al. [17].

**Pfmdr-1 and Pfcrf genotyping in isolates from day 0 and day of recurrence**

**Pfmdr-1** and **Pfcrf** genes were amplified in isolates from enrolment (and day of recurrence in cases of CQ treatment failure) by nested PCR approach, as described [12]. For **Pfmdr-1** gene, two fragments (549 bp, from codons 23 to 206 and 938 bp, from codons 966 to 1278) and for **Pfcrf** gene, three fragments (450 bp, from codon 37 in exon 2 to codon 125 in exon 3, 447 bp, from codon 121 in exon 3 to codon 211 in exon 4 and 850 bp, from codon 199 in exon 3 to codon 316 in exon 7) were sequenced. Sequencing reactions were carried out with ABI Prism BigDye Terminator cycle sequencing ready reaction kit and were run on a model 3730xl genetic analyzer (Applied Biosystems, Courtaboeuf, France). Electrophoregrams were visualized and analyzed with CEQ2000 genetic analysis system software (Beckman Coulter, Villepinte, France). Microsatellite PCR products were genotyped on the basis of unambiguous single allele signal at each nucleotide position.

**Pfmdr-1 copy number determination**

The **Pfmdr-1** copy number was measured by TaqMan real-time PCR (Rotor-Gene 6000, Corbett Research, Sydney, Australia) relative to the single copy of the β-tubulin (used as a housekeeping gene), as described [12]. All samples were run in duplicate in 23 μL reaction mixtures. For each run, the **Pfmdr-1** copy number was measured relative to the numbers in two standard calibrator parasite clonal lines (3D7 Africa and Dd2), by the ΔACT method [10]. Reference DNA clone W2 (which has three copies of **Pfmdr-1** gene) was used as the quality control in each run.

**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 Melhota 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 **Pfcrf** 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 **msp1**/**msp2** genotyping, (ii) wild-type **Pfcrf** 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 $H_e = \frac{n}{n(n-1)[1-p_i]}$ [n = the number of isolates sampled; $p_i$ = the frequency of the i<sup>th</sup> allele] [19]. Population genetic differentiation was measured using Wright’s F statistics [20]; population genetic parameters were computed with FSTAT software, v2.9.4 [21].

**Ethical approval**

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 ISRCTN36517353). 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 **Pfcrf** 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 IC\textsubscript{50} 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**

\textit{Pfmdr-1} alleles were determined for the 240 isolates from the \textit{in vivo} efficacy study (day 0 and day of recurrence, when available) and for 90 isolates for which the IC\textsubscript{50} for CQ was determined. Amongst \textit{in vivo} isolates, three of five major SNPs previously related to CQ-resistance were observed at day 0, N86\textit{Y} (147/240, 61.2%), Y184\textit{F} (172/240, 71.7%), and D1246\textit{Y} (80/240, 33.3%), present in eight different alleles: the wild-type allele NYD (11.2%), three single-mutant alleles [\textit{NFD}, 22.9%; \textit{YYD}, 14.6% and \textit{NYY}, 1.2%], three double-mutant alleles [\textit{YFD}, 77.9%; \textit{NFD}, 3.3% and \textit{YYF}, 1.2%] and one triple-mutant allele [\textit{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 \textit{YYD}, 8 single-mutant alleles \textit{NFD} and 5 double-mutant alleles \textit{YFD}). There was a non-random association between the N86\textit{Y} and D1246\textit{Y} loci ($R^2 = 0.23$, $P = 0.004$) and F184\textit{Y} and D1246\textit{Y} loci ($R^2 = 0.28$, $P = 0.0003$).

Amongst \textit{in vitro} isolates, only three isolates had an IC\textsubscript{50}>100 nM: one triple-mutant allele [\textit{YFY}, IC\textsubscript{50} = 142 nM], one double-mutant allele [\textit{NFD}, IC\textsubscript{50} = 126 nM] and one single-mutant allele [\textit{NFD}, IC\textsubscript{50} = 140 nM]. Comparison of geometric mean IC\textsubscript{50} 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 \textit{in vivo} studies and 71 from \textit{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.
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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 (NYD). 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-PiMSP-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 Pfndr-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-PiMSP-1 antibodies, gender, age, haemoglobin concentration at day 0, multiplicity of infection, presence of Pfndr-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 recurrence (OR = 4.6, 95%CI: 2.3–9.5, \( P < 0.0001 \)), age (OR = 1.2, 95%CI: 1.1 to 1.3, \( P = 0.0002 \)) and prevalence of antiPiMSP-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 Pfndr-1 allelic form at position 86 at day 1 (Pfndr-1 86N: 77.1%, n = 89 and Pfndr-1 86Y: 95.3%, n = 151, \( P = 0.0001 \)), day 7 (Pfndr-1 86N: 3.7%, n = 85 and Pfndr-1 86Y: 34.3%, n = 142, \( P < 0.0001 \)) and day 14 (Pfndr-1 86N: 2.0%, n = 78 and Pfndr-1 86Y: 54.9%, n = 129, \( P < 0.0001 \)) (Figure 3).

Cumulative incidence of recurrence of patients and Pfndr-1 86 mutation

The relation between Pfndr-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 Pfndr-1 86Y allele (\( n = 163 \), median time of recurrence 21 days) was significantly shorter than patients carrying Pfndr-1 86N allele (\( n = 77 \), median time of recurrence 28 days). The log-rank test shows that the two curves differ significantly (\( P < 0.0001 \) and the Pfndr-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%CI: 0.23–0.55).

Discussion

In the isolates from Madagascar studied here which harboured a wild type Pfct, Pfndr-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 Pfct gene and on additional mutations in Pfmdr-1 gene, which may increase the level of resistance afforded by Pfct. Pfct mutant alleles seem universally found, albeit with different haplotypes in different geographical regions [22]. The association of Pfndr-1 with CQ resistance was not found in some areas [10], possibly reflecting the fact that the impact of the Pfct/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 mains drugs used in Madagascar).

The observation that parasites with a wild Pfct 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 Pfct 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 Pfct 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 Pfct mutant alleles found in two sites from Madagascar (Andapa and Tsiroanomandidy) had a CVIET or CVIDT 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 Pfct 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 Pfndr1. A third possibility, is that the Pfct 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 Pfct 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 Pfct 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.

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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 P. falciparum came into play in these recrudescences. We did not detect any Pfmdr-1 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 recrudescences are not associated with selection of a minority, mutant Pfmdr-1 clone. The fourth hypothesis is that mutant alleles of Pfmdr-1 play a major role in CQ 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

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

| Exposure variables | Treatment outcome | P-value | OR | 95% CI |
|--------------------|-------------------|---------|----|--------|
|                    | Failed n | value | Cured n | value |
| Collection sites   | Tropical (reference) | 51 | 49.5% | 52 | 50.5% | 0.94* | - | - |
|                    | Highlands | 32 | 52.5% | 29 | 47.5% | 1.1 | 0.58–2.07 |
|                    | Sub-desert | 38 | 50.0% | 38 | 50.0% | 1.0 | 0.55–1.80 |
| EIR (number of bites of infected anophelines per person sleeping indoors) | <5 (reference) | 70 | 51.1% | 67 | 48.9% | 0.89 | - | - |
|                    | > =5 | 51 | 49.5% | 52 | 50.5% | 0.95 | 0.57–1.60 |
| Prevalence of anti-PfMSP-1 antibodies | <40% (reference) | 49 | 43.4% | 64 | 56.6% | 0.04 | - | - |
|                    | > =40% | 72 | 56.7% | 55 | 43.3% | 0.57 | 0.34–0.96 |
| Patient characteristics | Male gender | 121 | 44.3% | 119 | 50.4% | 0.2 | 0.78 | 0.47–1.30 |
|                    | Age (mean ± SD), year | 121 | 4.8 (3.7) | 119 | 7.03 (4.34) | <0.0001 | 0.88 | 0.83–0.94 |
|                    | 0.5–5 (reference) | 79 | 64.8% | 43 | 35.2% | - | - | - |
|                    | 6–10 | 33 | 39.5% | 49 | 60.5% | 0.0005* | 0.36 | 0.20–0.63 |
|                    | 11–15 | 11 | 28.9% | 27 | 71.1% | 0.002* | 0.22 | 0.10–0.49 |
| Temperature (°C) at day 0. mean ± SD | 121 | 38.4 (0.7) | 119 | 38.3 (0.8) | 0.61 | 1.0824 | 0.80–1.47 |
| Haemoglobin concentration (g/dL) at Day 0. mean ± SD | 121 | 9.4 (2.4) | 119 | 10.4 (2.1) | 0.0002* | 0.8 | 0.71–0.90 |
|                    | <10 | 73 | 61.3% | 46 | 38.7% | 0.001* | 2.36 | 1.41–3.96 |
|                    | > =10 (reference) | 49 | 40.1% | 73 | 59.9% | - | - | - |
| Previous CQ In-take (%) | 121 | 8.2 | 119 | 8.4 | 0.57* | 0.97 | 0.39–2.43 |
| Clinical isolates | Parasitaemia density at Day 0 (no.of parasites/μL) mean ± SD | 121 | 41,435 (60,928) | 119 | 43,289 (55,338) | 0.8* | 1 | - |
|                    | Multiplicity of infection (MOI) mean. ± SD | 121 | 1.9 (0.9) | 119 | 1.7 (0.8) | 0.06* | 0.39 | 0.98–1.96 |
| Pfmdr-1 86Y,% at Day 0 | 121 | 67.7% | 119 | 52.9% | 0.009* | 2.01 | 1.18–3.42 |
|                    | at Day of recrudescence | 121 | 82.6% | <0.0001* | 4.23 | 2.34–7.65 |
| Pfmdr-1 184F,% at Day 0 | 121 | 71.9% | 119 | 71.4% | 0.90 | 1.02 | 0.58–1.79 |
|                    | at Day of recrudescence | 121 | 81.0% | 0.08* | 1.70 | 0.93–3.11 |
| Pfmdr-1 1246Y. % at Day 0 | 121 | 31.3% | 119 | 31.9% | 0.98* | 0.99 | 0.50–3.11 |

*Treatment 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 Methods section; 
*Chi-squared test; 
†Fisher exact test; 
‡ANOVA test (or if P-value of the Bartlett’s test for inequality of population variances was < 0.05, Mann–Whitney/Wilcoxon two-sample tests); 
§OR: odds ratio; 
*95% Confidence Interval; 
¥Discordant alleles found in 16 isolates between day 0 (wild-type YD) and day of recrudescence (13 single-mutants YD and 3 double-mutants YFD); 
∗Discordant alleles found in 8 additional isolates between day 0 (wild-type NYD) and day of recrudescence (8 single-mutants NF D); 
†according to [18]; 
‡according to [19] and 
§according to [20]. 
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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 Pfct 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 Pfct mutant parasites was totally disconnected from the rate of clinical failures. Moreover, no selection of Pfct 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 N86Y 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) sexual 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 Pfct. This “soft” selection of Pfmdr-1 mutant alleles may also be due to quinine pressure, as quinine was for a long time and 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-N86Y 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 Pfct 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 antimalarials, 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.

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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 DM. Contributed reagents/materials/analysis tools: AR CB OMP RD DM. Wrote the paper: VA OMP RD DM.

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