RESEARCH

Molecular monitoring of *Plasmodium falciparum* drug susceptibility at the time of the introduction of artemisinin-based combination therapy in Yaoundé, Cameroon: Implications for the future

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Abstract

**Background:** Regular monitoring of the levels of anti-malarial resistance of *Plasmodium falciparum* is an essential policy to adapt therapy and improve malaria control. This monitoring can be facilitated by using molecular tools, which are easier to implement than the classical determination of the resistance phenotype. In Cameroon, chloroquine (CQ), previously the first-line therapy for uncomplicated malaria was officially withdrawn in 2002 and replaced initially by amodiaquine (AQ) monotherapy. Then, artemisinin-based combination therapy (ACT), notably artesunate-amodiaquine (AS-AQ) or artemether-lumefantrine (AL), was gradually introduced in 2004. This situation raised the question of the evolution of *P. falciparum* resistance molecular markers in Yaoundé, a highly urbanized Cameroonian city.

**Methods:** The genotype of *pfcrt* 72 and 76 and *pfmdr1* 86 alleles and *pfmdr1* copy number were determined using real-time PCR in 447 *P. falciparum* samples collected between 2005 and 2009.

**Results:** This study showed a high prevalence of parasites with mutant *pfcrt* 76 (83%) and *pfmdr1* 86 (93%) codons. On the contrary, no mutations in the *pfcrt* 72 codon and no samples with duplication of the *pfmdr1* gene were observed.

**Conclusion:** The high prevalence of mutant *pfcrt* 76T and *pfmdr1* 86Y alleles might be due to the choice of alternative drugs (AQ and AS-AQ) known to select such genotypes. Mutant *pfcrt* 72 codon was not detected despite the prolonged use of AQ either as monotherapy or combined with artesunate. The absence of *pfmdr1* multicoopies suggests that AL would still remain efficient. The limited use of mefloquine or the predominance of mutant *pfmdr1* 86Y codon could explain the lack of *pfmdr1* amplification. Indeed, this mutant codon is rarely associated with duplication of *pfmdr1* gene. In Cameroon, the changes of therapeutic strategies and the simultaneous use of several formulations of ACT or other anti-malarials that are not officially recommended result in a complex selective pressure, rendering the prediction of the evolution of *P. falciparum* resistance difficult. This public health problem should lead to increased vigilance and regular monitoring.

**Keywords:** Malaria, Cameroon, *pfcrt*, *pfmdr1*, *pfmdr1* copy number, Resistance, LNA probes

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Background
Monitoring the level of *Plasmodium falciparum* resistance against anti-malarial drugs is one of the keys to a successful malaria control. Although controlled clinical trials are the best available tool for assessing the relevance of anti-malarial treatments, molecular monitoring offers some advantages. Studies on single-nucleotide polymorphisms (SNPs) and duplication of genes involved in resistance can be carried out with more ease and are less time-consuming. Furthermore, molecular monitoring may reveal trends, allowing anticipation in the changes of treatment policies.

In Cameroon, the first-line recommended therapy for uncomplicated malaria was chloroquine (CQ) until 2002 and amodiaquine (AQ) monotherapy between 2002 and 2004. In January 2004, the artesunate-amodiaquine (AS-AQ) combination was officially adopted and artether-lumefantrine (AL) was added as an alternative artesinin-based combination therapy (ACT) in 2006 [1]. In practice, AS-AQ and AL have been used nationwide since 2007. AS-AQ is widely available in public health care centres while AL is relatively less prescribed because of its low supply in the public sector at a subsidized price [2].

The amplification of *pfmdr1* gene is a common molecular marker of mefloquine (MQ) resistance. An increase in the *pfmdr1* copy number is associated with clinical failures to MQ [3] and in vitro resistance to lumefantrine, which is an amino-alcohol, like MQ [4]. The amplification of *pfmdr1* gene has also been demonstrated to decrease the susceptibility to artesinin derivatives in the field as well as in laboratory-adapted *P. falciparum* strains [4-8]. Furthermore, a recent study in eastern Sudan reported an association between the carriage of parasites with increased *pfmdr1* copy number before treatment and recurrent parasitaemia after AL therapy [9].

In vitro, the *pfmdr1* N86 wild-type allele, independently of the *pfmdr1* copy number, is associated with a higher susceptibility to lumefantrine and MQ [3,10,11]. In parallel, in the field, *pfmdr1* N86 and *pfcr* K76 wild-type alleles were selected by artether-lumefantrine (AL) treatment whereas they were not selected by artesunate-amodiaquine (AS-AQ) or amodiaquine-sulphadoxine-pyrimethamine (AQ-SP) [12-15]. Conversely, the *pfmdr1* 86Y and *pfcr* 76T mutant alleles are associated with CQ resistance and also with AQ monotherapy failure [16-19]. Likewise, these haplotypes are selected by the association AS-AQ [20,21].

A *pfcr* genotype conferring high levels of resistance to AQ, corresponding to SVMNT haplotype of the codons 72-76, has been identified, first in Tanzania and more recently in Angola [22,23]. This haplotype, widely observed in Asia and South America, seems to be strongly selected by the use of AQ [24,25].

The objective of this study was to determine the prevalence of *pfmdr1* multiple copies and mutant *pfcr* 72 and 76 and *pfmdr1* 86 codons in Yaoundé, Cameroon at the time of the introduction of ACT. It is important to have a “molecular snapshot” of *P. falciparum* isolates at the beginning of this new anti-malarial therapeutic strategy, first, in order to make meaningful comparisons in the future and, secondly, to determine if there is any evidence of molecular mark suggesting a rapid evolution towards resistance.

Methods
Study sites and origin of samples
The study was carried out between 2005 and 2009, on a total of 447 samples from patients with a microscopy-confirmed diagnosis of uncomplicated *falciparum* malaria. The recruitment sites were in Yaoundé intra-muros (3° 52’ N, 11’ 31’ E), including the healthcare centre of Nkol- dongo (49 patients, median of three years old [0 month to 47 years]), the healthcare centre of Olembe (42 children, median of 2.5 years old [eight months to 12 years]), and the healthcare centre of Nlongkak (125 patients, median of two years old [six months to five years]). Two hundred and thirty-one samples were obtained from asymptomatic children aged from five to 11 years in Mfou (3°43’ N, 11° 38’E), 26 km from the centre of Yaoundé. This study was reviewed and approved by the Cameroonian National Ethics Committee and Cameroonian Ministry of Public Health.

Before patients with a positive thick smear have received an ACT treatment, finger-pricked capillary blood sample was collected on different filter papers, Whatman (Whatman plc, Middlesex, UK) or IsoCode STIX (Schleicher & Schuell, Keene, NH, USA). DNA from paper filters was extracted using the chlex-100 boiling method [26], concentrated by ethanol precipitation and frozen at -20°C until amplification.

Determination of *pfmdr1* copy number
To determine the copy number of *pfmdr1*, a qPCR method described previously was used [27]. All samples were tested in triplicate in 96-well plates on a LightCycler® 480 system (Roche Diagnostics, Neuilly sur Seine, France). Each run included two control DNA samples of reference *P. falciparum* clones, FCM29/Cameroon and Dd2/Indochina, which are known to have one and two-three copies of *pfmdr1* gene, respectively [27].

Genotyping of *pfcr* and *pfmdr1*
Genotyping of *pfcr* 76 and *pfmdr1* 86 codons was performed with a qPCR assay using Fluorescence Resonance Energy Transfer (FRET) hybridization probes and an analysis of the melting curve described previously [28,29]. Each run included two control DNA samples of *P. falciparum*: the CQ-susceptible F32/Tanzania strain with *pfcr* K76 and *pfmdr1* N86 wild-type alleles and the

References
[1] Keiser J, Utzschneider D, Utzschneider J, et al. Monitoring of molecular resistance markers in Plasmodium falciparum: the need for standardisation. Malar J 2008; 7:109.
[2] Welti C, Pinault S, Kiladjian J, et al. P. falciparum DNA in asymptomatic malaria carriers: a hidden reservoir? Malar J 2008; 7:110.
[3] Wellems TE, Craig AS, Krishna S, et al. Mefloquine resistance: a new molecular marker for clinical failures. Science 1991; 251:428–9.
[4] Blackwell T, Ayres S, Minakawa N, et al. Mutations in the multidrug resistance gene are associated with artesunate resistance in Papua New Guinea. Antimicrob Agents Chemother 2000; 44:3156–9.
[5] Craig AS, Krishna S, Wellems TE, et al. Detection of mefloquine resistance: a new PCR-based test using a TaqMan probe. Proc Natl Acad Sci USA 1999; 96:11276–81.
[6] World Health Organization. WHO guidelines for the treatment of uncomplicated malaria 2010. World Health Organization; 2010.
[7]WHO. WHO Guidelines for the Treatment of Malaria 2010. World Health Organization; 2010.
[8] WHO. WHO Guidelines for the Treatment of Malaria 2010. World Health Organization; 2010.
[9] Menard D, Schaefer PM, Verroust-Plantard C, et al. The association of artesunate and amodiaquine monotherapies and molecular markers of resistance in Cameroon, 2001-2004. Malar J 2008; 7:142.
[10] Menard D, Verroust-Plantard C, Merick J, et al. Impact of antimalarial treatments on parasite genotypes and drug resistance markers in Cameroon. PLoS ONE 2010; 5:e9047.
[11] Menard D, Verroust-Plantard C, Merick J, et al. Impact of antimalarial treatments on parasite genotypes and drug resistance markers in Cameroon. PLoS ONE 2010; 5:e9047.
[12] World Health Organization. WHO guidelines for the treatment of uncomplicated malaria 2010. World Health Organization; 2010.
[13] WHO. WHO Guidelines for the Treatment of Malaria 2010. World Health Organization; 2010.
[14] WHO. WHO Guidelines for the Treatment of Malaria 2010. World Health Organization; 2010.
[15] WHO. WHO Guidelines for the Treatment of Malaria 2010. World Health Organization; 2010.
[16] WHO. WHO Guidelines for the Treatment of Malaria 2010. World Health Organization; 2010.
[17] WHO. WHO Guidelines for the Treatment of Malaria 2010. World Health Organization; 2010.
[18] WHO. WHO Guidelines for the Treatment of Malaria 2010. World Health Organization; 2010.
CQ-resistant FCM29/Cameroon clone, carrying pfcr7 76T and pfdm1r 86Y mutant alleles.

The detection of the pfcr7 72S mutant allele was performed with a TaqMan probe-based genotyping assay the originality of which resides in the presence of Locked Nucleic Acid (LNA) inside the probes. LNA is a synthetic RNA analogue which, when integrated into an oligonucleotide, shows a strong affinity for their complementary targets [30]. Because of their thermal stability when hybridized to DNA, oligonucleotides containing LNA have a higher melting temperature (T_m) and could be used as primers, probes or clamps to improve molecular detection [31-33]. In general, sequences from P. falciparum contain a high percentage of adenine (A) and thymine (T) resulting in a low Tm and complicating molecular analysis. The introduction of LNA bases is a powerful tool to obtain discriminating probes with a moderate length and a probe hybridization that may occur during the annealing step in PCR. Consequently, this technique was particularly well suited for the experimental conditions described here. The pfcr7 gene was amplified by using primers P.falcA (5’-CAATT TTgTTTTAAgTTCTTTTAgCAA-3’) and P.falcF (5’-gTTTCtGTcTTTggTAAtACgTcCTA-3’). To genotype the different alleles, the amplified product was detected with one of the specific TaqMan probes: AF233067 probe, 5’-YAK-AATTgTATTCATT + A + C + ACTT + A + CA–BBQ-3’ hybridized with the pfcr7 72S mutant allele (SVMNT haplotype) and HM854027 probe, 5’-LC670-AATTgTTTTCAATT + A + C + ACATA + CA–BBQ-3’ hybridized with the pfcr7 C72 wild-type allele (CVIET haplotype) (the presence of a LNA nucleotide is preceded by the sign +). The primers and probes were designed in collaboration with Tib MolBiol Syntheselabor (Berlin, Germany). Master mixes contained 1 μl GeneAmp® 10 × PCR Gold Buffer (Applied Biosystems, Branchburg, NJ), 2.5 mM MgCl₂, 200 μM pooled dNTP, 500 nM of forward and reverse primers, 250 nM of each probe, 1 U per reaction of AmpliTaq® Gold DNA Polymerase (Applied Biosystems) and 2 μl of template DNA for a total reaction volume of 10 μl. Each reaction plate was run with control DNA samples of P. falciparum, in particular the 7GB/Brazil strain known to harbour the pfcr7 72S mutated allele [34], F32/Tanzania and FCM29/Cameroon as pfcr7 C72 wild-type control [28], water and DNA of healthy patient, which served as negative external amplification controls. The multiplex TaqMan assay reactions were carried out in a LightCycler® 480 Multiwell Plate 384 (Roche Diagnostics) with the following PCR programme: an initial step at 95°C for 12 minutes followed by 45 cycles of 10 seconds at 95°C and 45 seconds at 60°C. Data analysis for allelic discrimination was performed with the LightCycler® 480 software (Roche Diagnostics).

Statistical analysis

The proportions were compared using χ² test thanks to SigmaStat® software. The significance level (P) was fixed at 0.05.

Results

Pfdm1r copy number

The copy number of pfdm1r was determined for only 215 isolates from healthcare centres of central Yaoundé because of the limited amount of DNA samples from Mfou. Regardless of where the tested isolates were collected, none of them were identified with pfdm1r gene amplification (Table 1). The estimated gene copy number from all analysed isolates was close to 1, with an average copy number of 1.05 and a standard deviation of 0.20 (data not shown).

Pfdm1r and pfcr7 genotypes

The prevalence of pfdm1r and pfcr7 alleles in blood samples obtained from different sites in Yaoundé is presented in Table 1. The frequencies of the pfdm1r 86Y mutant genotype were 76% (153/201) and 84% (175/209) in Yaoundé and Mfou, respectively. Wild-type pfdm1r N86 genotype was observed in 10% (20/201) and 4% (9/209) of isolates, and 14% (28/201) and 12% (25/209) of isolates presented a mixed genotype in Yaoundé and Mfou, respectively. No significant differences were observed between Yaoundé and Mfou.

The frequencies of pfcr7 76T mutant genotype were 71% (145/203) and 55% (125/229), the pfcr7 K76 wild-type allele was present in 19% (38/203) and 15% (35/229) and mixed pfcr7 alleles occurred in 10% (20/203) and 30% (69/229) of the isolates in Yaoundé and Mfou, respectively, with a significant difference (p = 0.001).

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Contrary to pfcr7 76, a significant difference (p = 0.042) of the distribution of the alleles was observed between Yaoundé and Mfou when all samples with mixed pfdm1r 86 genotype are classified in mutant group.

No significant differences were observed either between the different healthcare centres of Yaoundé or between the different times of sample collection (data not shown).

None of the 414 samples tested for the codon 72 of pfcr7 gene was found with the mutant 72S allele (SVMNT haplotype).

Discussion

As elsewhere in the world, a very rapid development of resistance to anti-malarial drugs in Africa requires a regular monitoring in multiple and strategic points. With 53% of the population living in cities against 38% WHO African region, Cameroon is a highly urbanized African country [35]. This demonstrates the importance of
epidemiological studies in large cities such as Yaoundé, which currently has a population of over 1,800,000 inhabitants.

In the present study, a high prevalence of mutations associated with drug resistance was found in Yaoundé and its suburbs in both codon 76 of the pfcr7 gene (83%) and codon 86 of the pfmdr1 gene (93%) when all samples with mixed genotype were classified as mutant (Table 1). These results are in agreement with several other studies. Previous works of Basco et al. carried out in Yaoundé showed that 70% of 157 *P. falciparum* clinical isolates had the mutant pfcr7 76T codon in 2001 [36], and a large majority of isolates (88% of 64) carried the pfmdr1 86Y mutant allele between 1997 and 2000 [37]. Similarly, Mbacham et al. reported 77% and 76% prevalence of mutant pfcr7 76T and pfmdr1 86Y codons, respectively, in samples collected during the period 2004-2006 in Yaoundé [38]. Despite different classification of double populations and techniques with different sensitivity, the prevalence of mutations appears to increase (pfcr7) or remains at a high and relatively stable level (pfmdr1) until 2009 in spite of the official withdrawal of CQ from Cameroon in 2002. In some endemic areas, stopping the widespread use of CQ resulted in a return of chloroquine-sensitivity associated with the reappearance of wild-type genotypes. In the absence of drug pressure, *P. falciparum* wild-type haplotypes have a selective advantage over mutants. For example, in 1993, Malawi was the first sub-Saharan African country to replace CQ with SP nationwide in response to the high rates of CQ-resistant *falciparum* malaria. This change resulted in a decrease in the prevalence of the mutant pfcr7 haplotype associated with CQ resistance from 85% in 1992 to 13% in 2000. For pfmdr1 86Y mutant codon, the same study showed similar results but with lower amplitude (from about 60% in 1993 to around 20% in 2000) [39]. The recovery of CQ-sensitivity phenotype and genotype was also observed elsewhere in Malawi [40], Kenya [41] and China [42].

This stability of mutant pfcr7 76T and pfmdr1 86Y genotypes observed in Yaoundé and suburb may be the result of many factors. First of all, the choice of the replacement treatment logically influences the type of selected isolates. The use of SP, which has no influence on the selection of mutant pfcr7 and pfmdr1 genotypes, has been shown to favour, by a phenomenon of selective

| Genes and alleles | Number of samples (%) | Both sites | Yaoundé | Mfou (Suburb of Yaoundé) | P |
|------------------|-----------------------|-----------|---------|-------------------------|---|
| pfmdr1 amplification (nα = 215, nβ = NA) | | | | | |
| 1 pfmdr1 copy number | 215 (100%) | 215 (100%) | NA | NA | NA |
| > 1 pfmdr1 copy number | 0 (0%) | 0 (0%) | NA | NA | NA |
| pfmdr1 codon 86 (nα = 201, nβ = 209) | | | | | |
| Mutant 86Y haplotype only | 328 (80%) | 153 (76%) | 175 (84%) | NS |
| Mixed N86 and 86Y haplotypes | 53 (13%) | 28 (14%) | 25 (12%) | |
| Wild-type N86 haplotype only | 29 (7%) | 20 (10%) | 9 (4%) | |
| Total mutant 86Y haplotype | 381 (93%) | 181 (90%) | 200 (96%) | 0.042d |
| pfcr7 codon 76 (nα = 203, nβ = 229) | | | | | |
| Mutant 76T haplotype only | 270 (62%) | 145 (71%) | 125 (55%) | < 0.001t |
| Mixed K76 and 76T haplotypes | 89 (21%) | 20 (10%) | 69 (30%) | |
| Wild-type K76 haplotype only | 73 (17%) | 38 (19%) | 35 (15%) | |
| Total mutant 76T haplotype | 359 (83%) | 165 (81%) | 194 (85%) | NSd |
| pfcr7 codon 72 (nα = 202, nβ = 212) | | | | | |
| Mutant 72S haplotype | 0 (0%) | 0 (0%) | 0 (0%) | NS |
| Wild-type C72 haplotype | 414 (100%) | 202 (100%) | 212 (100%) | |

NA: not analysable because of the limited amount of DNA samples
NS: non significant
d: Number of total samples tested in the centre of Yaoundé
t: Number of total samples tested in the suburb of Yaoundé
<: Total mutant haplotype regroups single mutant haplotype and mixed haplotype, all samples with mixed genotype for the considered allele are classified in mutant group.
*: comparison of total mutant haplotypes versus wild-type haplotypes
*: comparison of mutant, wild-type and mixed haplotypes

Table 1 Frequency of mutations and/or gene amplification in anti-malarial resistance markers pfmdr1 and pfcr7 in Plasmodium falciparum isolates in Yaoundé and Mfou, Cameroon

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advantage, the reappearance of CQ-sensitive isolates harbouring wild-type pfcrt K76 and pfmdr1 N86 genotypes [39-41]. The use of AL or artesunate-mefloquine (AS-MQ) seems to favour the return to the predominance of wild-type pfmdr1 N86 genotype and, to a lesser extent, to the wild-type pfcrt K76 genotype by an active selection [14,43-45]. Inversely, AQ, a close Mannich base analogue of CQ, or AS-AQ promotes the maintenance of CQ-resistant isolates with the mutant pfcrt and pfmdr1 genotypes by an active selective pressure [20,46], as observed in the present study. Whereas in East African countries like Malawi or Kenya, SP or AL had largely replaced CQ [47], in Yaoundé, in 2005, AQ was still prescribed as a first-line anti-malarial drug in 20% and 63% of adults and children under five years old, respectively [2], and AS-AQ in 4.5% and 1.5%. AL was used only in 8.3% and 2.4%, AS-MQ in 1.5% and 0.8%, and SP in 5.8% and 0% of adults and children less than five years old, respectively [2].

Secondly, the changes of P. falciparum resistance phenotype and genotype after the withdrawal of CQ depend on the rapidity of drug replacement. For example, in Malawi where a profound and rapid return to CQ sensitivity was observed, the change in drug policy from CQ to SP was swift and efficient, so that SP became the only available anti-malarial drug in less than one year after the implementation of the new drug policy. In contrast, these changes were progressive and lasted several years in many areas as in Cameroon. In fact, in Yaoundé, although the National Malaria Control Programme of Cameroon had replaced CQ by AQ in 2002 and then AQ monotherapy by AS-AQ since January 2004, CQ was still largely accessible through the informal outlets (e.g. food market) in August 2005 [2].

Finally, in a more general way, fitness loss of mutant P. falciparum might be associated with the development of compensatory mechanisms able to maintain mutant parasites even in the absence of drug pressure [48]. This feature might explain, at least in part, the persistence of mutant pfcrt codon in Southeast Asia and South America [49-51] and also in Cameroon, as described here.

In Mfou, a higher frequency of mixed pfcrt haplotypes was observed at the expense of mutant pfcrt population. This observation was not done for pfmdr1 haplotypes. A possible reason for this observation is a drug pressure selection different from that existing in Yaoundé.

Since the probes used to detect the mutation in codon 76 of pfcrt gene were not able to detect that of codon 72, a new technique using LNA probes was developed in the present study to discriminate the mutant SVMNT haplotype (72S mutation) from the wild-type CVIET haplotype (C72 wild-type). Previous studies and data collected from countries like Bolivia or India suggested that AQ has an early and prominent role in the selection of parasites carrying SVMNT haplotype associated with drug resistance [24]. These parasites are highly resistant to AQ, but only moderately resistant to CQ. Contrary to CVIET haplotype, once the SVMNT haplotype emerges in a given parasite population and CQ and AQ are removed, the repopulation of sensitive strains may be very slow to occur [24]. As the SVMNT haplotype was recently described in Tanzania and Angola [22,23], it was important to verify whether this haplotype existed in Yaoundé. None of the samples tested for the codon 72 of pfcrt was found to carry the SVMNT haplotype. These results are contrary to what was observed in nearby African countries, such as in Ghana [52], Tanzania [22] and Angola [23] where the prevalence of this haplotype was between 3.9% and over 50%. It is possible that the observed predominance of SVMNT haplotype in Angola is the result of frequent travels of Brazilian and Angolan citizens between the two countries [23], which is not the case in Cameroon. However, the monitoring of pfcrt codons 72-76 should be pursued because AQ has long been prescribed in Cameroon before and since the cessation of the use of CQ (2002) and until 2005 [2] and seems to have an important role in the selection of the SVMNT haplotype [22].

The amplification of pfmdr1 gene has been more closely linked to MQ and halofantrine (HAL) resistance [53-55]. In this study, pfmdr1 amplification was not observed in Yaoundé between 2005 and 2009. Elsewhere in Africa, the situation seems to be contrasted. In various studies conducted in East Africa, only four samples were found with pfmdr1 gene duplication, one in Kenya and three in Sudan (near the Ethiopian border) among 475 isolates tested (57 in Sudan [9], 72 in Kenya [46], 186 in Zanzibar [53] and 160 in Malawi [54]). In West Africa, on the one hand, none of 580 samples tested in Liberia and Guinea-Bissau between 1981 and 2005 was found to be duplicated [55]; on the other hand, two studies had identified in Burkina Faso, Ivory Coast, Togo and Madagascar, six pfmdr1 duplications among 112 samples tested [27,56]. In Central Africa, data are limited since only 32 samples were screened and all of them had a single copy of pfmdr1 gene [27]. In this region, an exception is the study of Uhlemann et al who found the duplication of pfmdr1 gene in five of 62 clinical isolates tested (8%) in 1995 in Lambaréné, Gabon [57]. Four of these five patients harboured the wild-type N86 pfmdr1 codon even though during this period 90% of isolates carried the mutant pfmdr1 codon 86 around Lambaréné [58]. However, in 2002 at the same study site, none of 37 samples tested had pfmdr1 gene duplication. These observations on pfmdr1 gene amplification in Lambaréné are difficult to explain outside of the possible selection of such a clone by previous clinical trials on the same site, using low dose of mefloquine [58-60]. Nevertheless, these data showed that P. falciparum isolates from Central Africa can have pfmdr1 gene duplication.
The lack of pfmdr1 gene duplication in Yaoundé may possibly be due to the very low use of MQ or HAL, which represented only 1.5% of first-line treatments against malaria in 2005 [2], but also partly to the high prevalence of the pfmdr1 Y86 mutant allele. Indeed, in Southeast Asia, pfmdr1 amplification has been suggested to be incompatible in the presence of the mutant pfmdr1 86Y allele [61]. However, the conclusion of that Asian study has not been confirmed in Africa, where the existence of parasites harbouring a duplicated pfmdr1 gene with mutant 86Y codon has been reported from Sudan [9], Gabon [57] and Ivory Coast [27,56].

The molecular analysis performed in the present study did not find any pfcrt 72S mutation, which may be a good sign for the continued use of AQ in combination with AS. A regular evaluation of AS-AQ efficacy, in parallel with molecular surveillance, is required to ensure the utility of AS-AQ in Cameroon. This ACT contributes to the maintenance of a high prevalence of mutant pfcr 76T and pfmdr1 86Y alleles. The pressing question is to predict how these parasites will evolve in the presence of AL pressure. Several scenarios can be envisioned. Firstly, they could behave like Southeast Asian isolates and will not progress to the duplication of pfmdr1 gene in the absence of wild-type pfmdr1 N86 allele. Secondly, as already observed in some cases in Africa [9,27,56,57], the parasites may acquire multicopies of pfmdr1 despite the pfmdr1 86Y mutation. Only regular and exhaustive molecular monitoring of P. falciparum clinical isolates can provide the answer. However, the relevance of these results would be improved if they were associated with information on different antimalarial drugs that are really taken by the patients because these data often differ from the current national recommendation.

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Authors’ contributions
SM, PIM, carried out the molecular genetic studies. SM, AB, XI conceived and designed the study protocol and analysed the results. SM, AB, FBV, LKB drafted the manuscript. JFM, FBV, Xi participated in its design and helped to draft the manuscript. RT, CS, LKB and BL participated in study design, supervised clinical and laboratory diagnosis in the health care centres, and collected blood samples in Nlongkak, Olembe, and Nikondong, IM and PAA had the same role for the blood samples collected in Mfou. All authors read and approved the final manuscript.

Competing interests
The authors declare that they have no competing interests.

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References
1. WHO: World Malaria Report 2008, 2008 [http://www.who.int/malaria/publications/atoz/9789241563697/en/index.html].
2. Sayag C, Gaussené M, Vernazza-Licht N, Malvy D, Bley D, Millet P: Treatment of malaria from monotherapy to artemisinin-based combination therapy by health professionals in urban health facilities in Yaoundé, central province, Cameroon. Malar J 2009, 8:176.
3. Price RN, Uhlemann AC, Brockman A, McReddy E, Ashley E, Phaipun L, Patel R, Lai K, Locareesuwan S, White NJ, Nosten F, Krishna S: Mefloquine resistance in Plasmodium falciparum and increased pfmdr1 gene copy number. Lancet 2004, 363:438-447.
4. Sidhu AB, Uhlemann AC, Valderramos SG, Valderramos JC, Krishna S, Fidock DA: Decreasing pfmdr1 copy number in Plasmodium falciparum malaria heightens susceptibility to mefloquine, lumefantrine, halofantrine, quinine, and artemisinin. J Infect Dis 2006, 194:528-535.
5. Lim P, Aller AP, Khim N, Shah NK, Incardona S, Dongu S, Yi P, Bough D, Bouchier C, Pujolion OM, Meshnick SR, Wongschikanalai C, Fandeur T, Le Bras J, Ringwald P, Arey F: Pfmdr1 copy number and artemisinin derivatives combination therapy failure in falciparum malaria in Cambodia. Malar J 2009, 8:11.
6. Carrara VI, Zhang J, Ashley EA, Price RN, Steppenwolks K, Barends M, Brockman A, Anderson T, McReddy E, Phaipun L, Proux S, Van Vugt M, Hutygalung R, Maung Lwin K, Pyae Phyo A, Preechapornkul P, Fandeur T, Le Bras J, Ringwald P, Arey F: Pfmdr1 copy number and artemisinin derivatives combination therapy failure in falciparum malaria in Cambodia. Malar J 2009, 8:11.
7. Teuscher F, Gatton ML, Chen N, Peters J, Kyle DE, Cheng Q: Artemisinin-induced dormancy in Plasmodium falciparum: duration, recovery rates, and implications in treatment failure. J Infect Dis 2010, 202:1362-1368.
8. Gadalla NB, Adam J, Elbak SE, Bashir S, Mukhtar I, Oguke M, Gadalla A, Mansour F, Wathur D, El-Sayed BB, Sutherland CJ: Increased pfmdr1 Copy Number and Sequence Polymorphisms in Plasmodium falciparum
Isolates from Sudanese Malaria Patients Treated with Artemether-Lumefantrine. Antimicrob Agents Chemother 2011, 55:5408-5411.

10. Siddhu AB, Valderramos SG, Flock DA: pfmdr1 mutations contribute to quinine resistance and enhance mefloquine and artesinin sensitivity in Plasmodium falciparum. Mol Microbiol 2005, 57:913-926.

11. Duraisingh MT, Jones P, Sambou I, von Seidlein L, Pinder M, Warhurst DC: The tyrosine-86 allele of the pfmdr1 gene of Plasmodium falciparum is associated with increased sensitivity to the anti-malarials mefloquine and artesinin. Mol Biochem Parasitol 2000, 108:13-23.

12. Martensson A, Stromberg J, Siowath C, Maellem M, Gil JP, Montgomery SM, Olliaro P, Ali AS, Bjorkman A: efficacy of artesunate plus amodiaquine versus that of artemether-lumefantrine for the treatment of uncomplicated childhood Plasmodium falciparum malaria in Zanzibar, Tanzania. Clin Infect Dis 2005, 41:1079-1086.

13. Bairaine FN, Rosenthal PJ: Prolonged selection of pfmdr1 polymorphisms after treatment of falciparum malaria with artemether-lumefantrine in Uganda. J Infec Dis 2011, 204:1120-1124.

14. Siowath C, Stromberg J, Martensson A, Maellem M, Ollbondo C, Bjorkman A, Gil JP: In vivo selection of Plasmodium falciparum pfmdr1 86 N coding alleles by artemether-lumefantrine (Coartem). J Infect Dis 2005, 191:1014-1017.

15. Some AF, Seré Y, Dokomajilar C, Zongo I, Rouamba N, Greenhouse B, Ouedraogo JB, Rosenthal PJ: Selection of known Plasmodium falciparum resistance-mediating polymorphisms by artemether-lumefantrine and amodiaquine-sulfadoxine-pyrimethamine but not dihydroartemisinin-piperaquin in Burkina Faso. Antimicrob Agents Chemother 2010, 54:1949-1954.

16. Ochong EO, van den Broek IV, Neus K, Nzila A: Short report: association between chloroquine and amodiaquine resistance and allelic variation in the Plasmodium falciparum multiple drug resistance 1 gene and the chloroquine resistance transporter gene in isolates from the upper Nile in southern Sudan. Am J Trop Med Hyg 2003, 69:184-187.

17. Holmgren G, Gil JP, Ferreira PM, Vega MI, Oubono CO, Bjorkman A: Amodiaquine resistant Plasmodium falciparum malaria in vivo is associated with selection of pft76 and pfmdr1 86T. Infect Genet Evol 2006, 6:309-314.

18. Hopp CT, Gotohso GO, Folarin OA, Bolaji OM, Sawummi A, Kyle DE, Milhouse W, Wirth DF, Oduola AM: Association between mutations in Plasmodium falciparum chloroquine resistance transporter and P. falciparum multidrug resistance 1 genes and in vivo amodiaquine resistance in P. falciparum malaria-infected children in Nigeria. Am J Trop Med Hyg 2007, 75:153-161.

19. Tinto H, Guellouz L, Zongo I, Guiguemde RT, D’Alessandro U, Ouedraogo JB: Chloroquine-resistance molecular markers (PfCRT T76 and Pfmdr1 Y86) and amodiaquine resistance in Burkina Faso. Trop Med Int Health 2010, 13:238-240.

20. Djimde AA, Fofana B, Sagara I, hireder S, Dembele D, Dama S, Ouologuem D, Dicko A, Doumbo OK: Amodiaquine resistant Plasmodium falciparum malaria in vivo is associated with selection of pft76 and pfmdr1 86T. Infect Genet Evol 2006, 6:309-314.

21. Wang X, Mu J, Li G, Chen P, Guo X, Fu L, Chen L, Su X, Wellems TE: Decreased prevalence of the Plasmodium falciparum chloroquine resistance transporter 76T marker associated with cessation of chloroquine use in Malawi. J Infect Dis 2007, 195:1870-1875.

22. Mita T, Kaneko A, Lum JK, Bwogo B, Takei M, Zungu L, Tsukahara T, Tanabe K, Kobayakawa T, Bjorkman A: Recovery of chloroquine sensitivity and low prevalence of the Plasmodium falciparum chloroquine resistance transporter gene mutation K76T following the discontinuation of chloroquine use in Malawi. J Infect Dis 2007, 196:413-415.

23. Wang X, Mu J, Li G, Chen P, Guo X, Fu L, Chen L, Su X, Wellems TE: Decreased prevalence of the Plasmodium falciparum chloroquine resistance transporter 76T marker associated with cessation of chloroquine use against P. falciparum malaria in Hainan, People’s Republic of China. Am J Trop Med Hyg 2007, 77:410-414.

24. Siowath C, Petersen I, Veiga MI, Martensson A, Premji Z, Bjorkman A, Flock DA, Gil JP: In vivo selection of Plasmodium falciparum parasites carrying the chloroquine-susceptible pfk76 allele after treatment with artemether-lumefantrine in Africa. J Infect Dis 2009, 199:750-757.

25. Yang Z, Zhang Z, Sun X, Wan W, cui L, Zhang X, Zhong D, yan G, Cui L: Molecular analysis of chloroquine resistance in Plasmodium falciparum in Yunnan Province, China. Trans Med Int Health 2007, 12:1051-1060.
45. Lekana-Douki JB, Drnouza Boutamba SD, Zatra R, Zang Edou SE, Ekomy H, Blessigou U, Touré-Ndouo FS. Increased prevalence of the Plasmodium falciparum pfmdr1 86N genotype among field isolates from Franceville, Gabon after replacement of chloroquine by artemether-lumefantrine and artesunate-mefloquine. Infect Genet Evol 2011, 11:512-517.

46. Holmgren G, Bjorkman A, Gil JP. Amodiaquine resistance is not related to rare findings of pfmdr1 gene amplifications in Kenya. Trop Med Int Health 2006, 11:1808-1812.

47. Lauer MW, Plowe CV. Withdrawing antimalarial drugs: impact on parasite resistance and implications for malaria treatment policies. Drug Resist Updat 2004, 7:279-288.

48. Walliker D, Hunt P, Babiker H. Fitness of drug-resistant malaria parasites. Acta Trop 2005, 94:251-259.

49. Cortesse JF, Caraballo A, Contreras CE, Plowe CV. Origin and dissemination of Plasmodium falciparum drug-resistance mutations in South America. J Infect Dis 2002, 186:999-1006.

50. Vieira PP, Das Gracas Alecrim M, Da Silva LH, Gonzalez-Jimenez I, Zalis MG. Analysis of the pfcrt K76T mutation in Plasmodium falciparum isolates from the Amazon region of Brazil. J Infect Dis 2001, 183:1832-1833.

51. Zalis MG, Pang L, Silveira MS, Milhous WK, Wirth DF. Evidence for quinine resistance. Am J Trop Med Hyg 1998, 58:630-637.

52. Mehlotra RK, Mattera G, Bockarie MJ, Maguire JD, Baird JK, Sharma YD, Allfrangs M, Dorsey G, Rosenthal PJ, Fryauff DJ, Kazura JW, Stoneking M, Zimmerman PA. Discordant patterns of genetic variation at two chloroquine resistance loci in worldwide populations of the malaria parasite Plasmodium falciparum. Antimicrob Agents Chemother 2008, 52:2212-2222.

53. Snowath C, Ferreira PE, Bustamante LY, Dahlstrom S, Martensson A, Bjorkman A, Krishna S, Gil JP. The role of pfmdr1 in Plasmodium falciparum tolerance to artemether-lumefantrine in Africa. Trop Med Int Health 2007, 12:736-742.

54. Nkhoma S, Nair S, Mukaka M, Molyneux ME, Ward SA, Anderson TJ. Parasites bearing a single copy of the multi-drug resistance gene (pfmdr-1) with wild-type SNPS predominate amongst Plasmodium falciparum isolates from Malawi. Acta Trop 2009, 111:78-81.

55. Ursing J, Kofoed PE, Rombo L, Gil JP. No pfmdr1 amplifications in samples from Guinea-Bissau and Liberia collected between 1981 and 2004. J Infect Dis 2006, 194:716-718, author reply 718-719.

56. Basco LK, Le Bras J, Rhoades Z, Wilson CM. Analysis of pfmdr1 and drug susceptibility in fresh isolates of Plasmodium falciparum from subsaharan Africa. Mol Biochem Parasitol 1995, 74:157-166.

57. Uhlemann AC, Ramharter M, Lelli B, Kremsner PG, Krishna S. Amplification of Plasmodium falciparum multidrug resistance gene 1 in isolates from Gabon. J Infect Dis 2005, 192:1830-1835.

58. Mawili-Mboumba DP, Kun JF, Lelli B, Kremsner PG, Ntoumi F. Pfmdr1 alleles and response to ultralow-dose mefloquine treatment in Gabonese patients. Antimicrob Agents Chemother 2002, 46:166-170.

59. Radloff PO, Philips J, Nkeyi M, Sturchler D, Mittelholzer ML, Kremsner PG. Artemether compared with mefloquine for treating Plasmodium falciparum malaria in children. Am J Trop Med Hyg 1996, 55:259-262.

60. Lelli B, Lehman LG, Schmidt-Ött JR, Sturchler D, Handschin J, Kremsner PG. Malaria chemotherapy trial at a minimal effective dose of mefloquine/sulfadoxine/pyrimethamine compared with equivalent doses of sulfadoxine/pyrimethamine or mefloquine alone. Am J Trop Med Hyg 1998, 58:619-624.

61. Woodrow CJ, Krishna S. Antimalarial drugs: recent advances in molecular determinants of resistance and their clinical significance. Cell Mol Life Sci 2006, 63:1586-1596.