Emergence of Resistance to Atovaquone-Proguanil in Malaria Parasites: Insights From Computational Modeling and Clinical Case Reports

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ABSTRACT

The usefulness of atovaquone-proguanil (AP) as antimalarial treatment is compromised by the emergence of atovaquone resistance during therapy. However, the origin of the parasite mitochondrial DNA (mtDNA) mutation conferring atovaquone resistance remains elusive.

Here, we report a patient-based stochastic model that tracks the intra-host emergence of mutations in the multi-copy mtDNA during the few erythrocytic parasite cycles leading to the malaria febrile episode. The effect of mtDNA copy number, mutation rate, mutation cost and total parasite load on the mutant parasite load per patient was evaluated.

Computer simulations showed that almost any infected patient carried, after 4 to 7 erythrocytic cycles, de novo mutant parasites at low-frequency, with varied frequencies of parasites carrying varied numbers of mutant mtDNA copies. A large inter-patient variability in the size of this mutant reservoir was found, explained by the different parameters tested but also by the relaxed replication and partitioning of mtDNA copies during mitosis.

We also report seven clinical cases in which AP-resistant infections were treated by AP. These provided evidences that parasiticidal drug concentrations against AP-resistant parasites were transiently obtained within days following treatment initiation.

Altogether, these results suggest that each patient carries new mtDNA mutant parasites emerging before treatment and that are destroyed by high starting drug concentrations; however, because the size of this mutant reservoir is highly variable from patients to patients, we propose that some patients fail to eliminate all the mutant parasites, repeatedly producing de novo AP treatment failures.

INTRODUCTION

The recent emergence of *Plasmodium falciparum* malaria resistance to artemisinins in Southeast Asia may lead to major public health consequences in a close future. There is an urgent need to identify treatment regimens that maximize the therapeutically useful life span of current antimalarial drugs (1). Among possible non-ACT available alternatives the role of atovaquone-proguanil (AP; initially licensed as Malarone©) has been questioned (2-4). Atovaquone-proguanil is a popular prophylactic drug and also shows high efficacy in the treatment of uncomplicated *falciparum* malaria in travellers. Recently it has been used in the Greater Mekong Subregion to treat individuals detected as *P. falciparum* carriers as part of a plan to contain artemisinin resistance (4). In addition, generic forms of Malarone© have been recently licensed, and the marketing and use of AP could increase in endemic settings (5).

Here, we focused on atovaquone resistance which develops easily during the treatment of uncomplicated *P. falciparum* malaria using atovaquone as monotherapy (6). This high failure rate prompted the introduction of the atovaquone-proguanil (AP) combination therapy. Atovaquone is a potent inhibitor of the cytochrome *bc1* complex or complex III in the mitochondrial electron transport chain (7). The addition of proguanil significantly increases the ability of atovaquone to collapse the mitochondrial membrane potential (8), and also generates in vivo the metabolite cycloguanil which antagonizes the parasite dihydrofolate reductase (dhfr) in the folate pathway (9-10). Resistance of *P. falciparum* to atovaquone is primarily determined by one point mutation in the mitochondrial and multi-copy *cytochrome b* (*cytb*) gene (11-12) which encodes a subunit of the cytochrome *bc1* complex. In addition, *P. falciparum* parasites harbouring *dhfr* mutations exhibit cross resistance to both antifolates pyrimethamine and cycloguanil (9, 13-14). Nowadays, a high rate of antifolate resistance is found in *P. falciparum* parasites across major endemic areas (15). Hence, infecting parasites often carry antifolate resistance and just a single evolutionary step –the acquisition of the *cytb* mutation– is sufficient to make parasites resistant to both cycloguanil and atovaquone. Despite that the atovaquone resistance mutation is not detected in endemic areas (16-18), about 1 in 100 non-immune travellers returning to the UK and who took AP as treatment to cure a *P. falciparum* malaria episode had treatment failure associated with atovaquone resistance (19). Genetic studies suggest that the atovaquone resistance mutation evolves *de novo* during the primary infection or its treatment, being then selected by AP treatment (20-22).

However, the mechanism underlying the rapid evolution of the *cytb* mutant parasites during AP treatment remains elusive. Various hypotheses suggest a role for an increased rate of mitochondrial DNA (mtDNA) mutations induced by atovaquone (23) and for pharmacokinetic-phamacodynamic considerations (5). Here another explanation was considered for the intra-host acquisition of atovaquone resistance. We hypothesize that the multiple mtDNA copies per parasite along with the complex replication of mtDNA (24-25) could favour a large mutational input before treatment. Addressing experimentally the intra-host dynamics of mtDNA mutations is currently not possible in

the *P. falciparum* model, mainly because of its intractability, and modeling offers an opportunity to explore this question.

Therefore, we developed for the first time a patient-based stochastic model that tracks the intra-host emergence of malarial mtDNA mutations during the few rounds of asexual blood stage reproduction leading to the malaria febrile episode and diagnosis. Briefly, it describes the appearance of spontaneous mtDNA mutations and their propagation within replicating mitochondrial genomes and parasites according to intra- and inter-cellular random drift, in absence of drug selection. We evaluated the effect of several parameters on the intra-host frequency of mtDNA-mutated parasites: the mtDNA mutation rate, the fitness cost associated to the mtDNA mutation, the number of mtDNA copies per parasite, and the total parasite load per patient. Then, we reported clinical evidences showing for the first time how AP-resistant parasites responded to AP treatment. Finally, we integrated these computational and clinical new findings to propose a mechanistic framework describing how atovaquone resistance emerges during malaria blood stages and how it translates into AP treatment failure, and we discuss the implications of these results for drug usage in the field.

MATERIAL AND METHODS

We used a two-stage process: 1) we developed a model to calculate the probability that new mtDNA mutations will be present among the malaria parasites at the time of treatment initiation; and 2) we evaluated whether any such mtDNA-mutated parasites could survive AP treatment and recrudesce as a new resistant infection. This two steps strategy follows a similar logic as the one used for nuclear genes encoding resistance (26).

1) The model. The mathematical description of the model is provided in the Supplementary Text.

Model framework. The model simulates the replication and partitioning of parasitic mitochondrial genomes within a replicating blood stage parasite lineage. It tracks the production and propagation of parasite mtDNA mutations within a single infected human host, starting from the delivery of hepatic parasitic forms into the blood to the time at which the patient will seek for medical care and treatment. We assumed that: the infection takes place in a non-immune host; all parasites delivered from hepatocytes into the blood have the wild type mitochondrial genotype; the mutation is a single nucleotide replacement that arises through random and recurrent mtDNA replication errors during the erythrocytic asexual parasite growth.

Parasite growth. We assumed that all initial parasites originated from a single release from the liver and that each parasite infected a single erythrocyte. In each infected erythrocyte, the nuclear and mitochondrial genomes of the parasite each are replicated through five consecutive endo-mitosis (within one parasite cell) during one erythrocytic 48h-cycle, eventually leading to 32 daughter parasites. Upon egress from the erythrocyte, each daughter parasite released in the blood has a probability p_surv to survive and initiate a new erythrocytic cycle. We assumed parasite growth and cycles to be synchronous within a single host infection.

Replication and partitioning to daughter cells of mitochondrial genomes. The detailed mechanisms by which malaria mitochondrion and its genome replicate and are passed to daughter cells are largely unknown, although important features have been described (24, 27). Hence we used a sequence of events with minimum mechanistic assumptions. Eukaryotic organelle genomes, such as mtDNA, undergo *relaxed* replication and partitioning, as compared to the stringent nuclear genome (25, 28); it means that multiple mtDNA copies are replicated and partitioned randomly, with respect to genotype.

We assumed that: i) each parasite contains one mitochondrion (29); ii) one mitochondrion contains n copies of its genome (24); iii) n is constant within an infection; iv) at each mtDNA replication, the template copies to be duplicated will be selected by n random draws with replacement – allowing some mtDNA copies to be replicated several times while other copies not to be replicated. This leads to a parasite containing one mitochondrion with 2n mtDNA copies; v) the mitochondrion containing 2n mtDNA copies is then partitioned into two independent mitochondrial units, each containing n mtDNA copies selected at random among the 2n mtDNA copies; vi) the duplication/partitioning cycle of the mitochondrial genome repeats five times during one erythrocytic

48h-cycle; then, each of these 2^5=32 mitochondrial units will constitute the single mitochondrion of one of the 32 daughter parasites; vii) the resistance mutation arises spontaneously in one mtDNA copy with a probability μ (per nucleotide per replication); because the atovaquone resistance mutation is a single nucleotide substitution, we assumed that the probability of a resistant mutation to appear equals the per nucleotide mitochondrial mutation rate ; during the course of the erythrocytic phase, n different mutant parasite genotypes can be produced within a single host: parasites having from 1 to n of their n mtDNA copies mutated.

We did not consider back mutation because its effect is unlikely to alter significantly the intra-host frequency of the resistance mutation, specifically on the short time scale that our study addresses (1 to 7 cycles of 48hr intra-erythrocytic growth).

Parasite definition according to mitochondrial genotype. i) parasites having none of their n mtDNA copies mutated were defined as *wild type* ; ii) parasites having from 1 to n-1 of their n mtDNA copies mutated were defined as *heteroplasmic*; iii) parasites having all of their mtDNA copies mutated were defined as *homoplasmic-mutant*; iv) the grouping of both heteroplasmic and homoplasmic-mutant parasites was defined as *all-mutant* parasites.

Mutation cost and fitness. In some simulations, we assumed that, in absence of drug, carrying the resistance mutation results in decreased parasite fitness (12, 30-31). We defined the fitness as the proportion of daughter parasites of a given mitochondrial genotype that will succeed in invading new erythrocytes. However, the mutation does not alter the probability of a mutant mtDNA copy to be replicated or partionned (25). In addition to the neutral situation (no decrease in fitness associated to the mutation), two other situations were considered. First, the mutation is recessive relative to the decrease in fitness: only homoplasmic-mutants have a decreased survival probability when compared to any other genotype. Second, the mutation is dominant relative to the decrease in fitness: the survival probabilities of heteroplasmic and homoplasmic-mutants are the same, and lower than the one of wild type parasites. The recessive and dominant effects explored here represent the two ends of a spectrum, in which the parasite fitness would be a function of the number of mutated mitochondrial genes.

Input parameters. i) the number of parasites delivered into the blood after the hepatic phase: $x = 5x10^4$; ii) the mtDNA mutation rate: $\mu = 10^{-9}$, 10^{-10}, and10^{-11}/nucleotide/replication. To our knowledge, there is no direct and unbiased estimate for the probability of a mtDNA change per nucleotide per mtDNA replication in *P. falciparum*. Therefore, we have used very conservative values based on values estimated in other species which range from $0.7x10^{-8}$ to $17x10^{-8}$/nucleotide/mtDNA replication (with one DNA replication/cell division (32-35)). In *P. falciparum* the nuclear mutation rate was estimated to be $1.7x10^{-9}$/nucleotide/48hr-generation during the blood stage growth (36); iii) the parasite survival probability associated with the distinct mitochondrial genotypes (12, 37): wild type, p_surv = 12/32; homoplasmic-mutant, p_surv_m = 6/32; heteroplasmic, p_surv_ht = 12/32 when the mutation cost is recessive and p_surv_ht = 6/32 when it is dominant; iv) the number of mtDNA copies/parasite: n = 5, 10, 20, and 40. This copy number was estimated to be about 20

183 copies/cell for *P. falciparum*, based on the analysis of a single parasite strain (24); v) the number of
184 erythrocytic 48h-cycles following delivery from the liver: from 1 to 7 (this corresponds from 2 to 14
185 days after the hepatic phase ; the median delay between onset of symptoms and treatment is 4 days in
186 imported *falciparum* malaria (38)).
187 **Model simulations.** Varying parameters were the mtDNA copy number, the mutation rate, the
188 mutation cost, and the number of erythrocytic 48h-cycles following parasite delivery from the liver.
189 Other parameters were fixed. For each combination of parameters tested, 20,000 simulations were run.
190 The simulations have been performed with R v 2.12.2 (R Foundation for Statistical Computing,
191 Vienna, Austria).
192 **2) Clinical case reports.** The French malaria reference center database was searched for late AP
193 treatment failures in which the recrudescent infections were treated again with AP. A total of 6,247
194 imported *P. falciparum* infections diagnosed and treated in France with AP were recorded by the
195 French malaria reference center, 2002-2012. Basic demographic and epidemiologic data, clinical and
196 parasitological informations, treatment, and history of travel and of malaria infections were collected
197 systematically. Pre- and post-treatment blood isolates were sent to the French malaria reference center
198 by French hospitals participating in the sentinel network for drug resistance and plasma drug testings.
199 Analyzed samples were obtained by blood collections required by the standard medical care for all
200 patients presenting with fever upon hospital admission in France.
201 **3) Ethics statement.** According to the French legislation, bio-banking and secondary use for scientific
202 purpose of human clinical remaining samples are possible as long as the corresponding patients are
203 informed and has not given any objection to them. Here, this requirement was fulfilled as the
204 information is given to every patient through the Hospital notice entitled “Information for patients”,
205 and no immediate or delayed patient opposition was reported by the hospital clinicians to the French
206 malaria reference center. Moreover, samples received at the French malaria reference center have been
207 registered and declared for research purpose as a bio-bank to both the Assistance Publique des
208 Hôpitaux de Paris and to the French National Insitute of Health Survey. No institutional review board
209 approval is required according to the French legislation (article L. 1111-7 du Code de la Santé
210 Publique, article L. 1211-2 du Code de Santé Publique, articles 39 et suivants de la loi n° 78-17 du 6
211 janvier 1978 modifiée en 2004 relative à l’informatique, aux fichiers et aux libertés). Samples used
212 were not anonymized.
213 **4) Molecular analysis.** Parasite genomic DNA was extracted from whole blood, thin or thick blood
214 smears, and plasma. The *cytb* gene was sequenced directly from PCR products to genotype the codon
215 $cytb^{268}$ that associates with atovaquone resistance. Five nuclear microsatellite locus (TAA81, TAA87,
216 PfPK2, Ara2, and TAA60) dispersed on 5 different *P. falciparum* chromosomes were genotyped by
217 capillary electrophoresis to determine the nuclear genetic background associated with each of these
218 parasite isolates. All methods were as previously described (22). The codons 51, 59 and 108 of the

dhfr gene associated with cycloguanil resistance were genotyped either by PCR-RFLP as described in (39) or by direct sequencing of PCR products.

5) Estimation of the parasite reduction ratio for atovaquone-proguanil/cycloguanil-resistant parasites. To evaluate the intrinsic *in vivo* efficacy of AP treatment on drug resistant parasites, we measured the parasite reduction ratio (PRR) which is defined as the fractional reduction in parasite number per asexual life cycle (i.e. 48h) or time unit (day) during the treatment (40). The parasite reduction ratio is similar to the killing rate induced by a specific treatment. A parasite reduction ratio of 50 means that 1 out 50 parasites survive to drug treatment per asexual life cycle. Based on the clinical cases in which infected patients carrying AP-resistant parasites were treated with AP and for which two non-negative and consecutive blood parasite counts were recorded, we estimated the PRR as being equal to $(P_0/P_t)^{2/t}$, where P_0 and P_t are parasite loads at days 0 and t, respectively (41-42).

6) Estimation of the minimum time required to clear all the cryptic *de novo* mutant parasites from an infection. Using the intra-host distribution of mtDNA-mutated parasites generated through 20,000 simulated infections, we estimated the minimum time (T, in days) during which blood drug concentrations should equal or exceed the minimum parasiticidal concentration (MPC^{APR}) to clear all the cryptic mtDNA-mutated parasites. We assumed that the PRR of AP-resistant parasites (PRR^{APR}) is constant and that AP-resistant parasites are killed at the same rate whether they represent the minority or the dominant population within the infection. An estimate for T can be obtained using the following formula: $T = 2 \times \ln B_m / \ln(PRR^{APR})$, where B_m is the mutant parasite load per infection (41). We estimated T for simulations obtained with a recessive fitness cost, copy number = 20 and mutation rate = 10^{-10}/nucleotide/replication.

RESULTS

A model of mtDNA evolution for the pre-treatment phase. We modeled the evolution of *de novo* mtDNA mutations that arose during the intra-host expansion of replicating *P. falciparum* erythrocytic forms in the absence of drug selection. After the delivery of parasites from the liver into the blood stream, the mean total parasite load per patient multiplied by ~12-times/erythrocytic 48hr-cycle and reached about $1.5x10^{11}$ parasites at the 6th cycle (corresponding to 12 days after the hepatic phase). Assuming a volume of 5 liters of blood per patient and an erythrocyte concentration of $5x10^{12}$/L, this translates to 0.6% of total host erythrocytes being infected. During this intense replicating phase, random recurrent mtDNA mutations occurred and then replicated and segregated according to random drift (combined or not with negative selection) within daughter mitochondrial genomes and parasites (Fig. 1). This produced heteroplasmic parasites and ultimately homoplasmic-mutant parasites, having all of their mtDNA copies being mutated (Fig. 1A). For our reference model (neutral mutation, 20 mtDNA copies per parasite, and a mtDNA mutation rate of 10^{-10}/nucleotide/replication), all-mutant parasites (having at least one mtDNA copy being mutated) appeared on average at the first erythrocytic cycle with a mean frequency of ~ $4x10^{-9}$ all-mutant parasites/infection which then slightly increased with additional 48hr-cycles to reach ~75 times the mutation rate at the 6th cycle (Fig. 1B). Homoplasmic-mutant parasites appeared on average at the 3rd cycle and then increased in frequency to a mean value close to the mutation rate at the 6th cycle (Fig. 1B). At the 6th cycle the composition of the mtDNA mutant parasite population per patient was found to be complex, each patient harbouring at varied and low frequencies an array of genotypically distinct mutant parasites defined by the number of mtDNA copies being mutated (Fig. 1C and 1D). Next, we focused on the intra-host distribution of mutant parasites at the 6th cycle when the mean total parasite load per patient was ~$1.5x10^{11}$. The number of mtDNA copies per parasite had opposite effects on the intra-host distribution of homoplasmic- and all-mutant parasite load (Fig. 2A and 2B): the larger the copy number, the larger is the number of all-mutants but the lower is the number of homoplasmic-mutants per infection. The size of both the homoplasmic- and all-mutant populations increased with the mutation rate (Fig. 2C and 2D) and with the total parasite load per patient (Fig. 2E and 2F). Finally, the fitness cost of the resistance mutation altered the number of mutant parasites per infection in a very expected way when compared to a neutral model (Fig. 2G and 2H): a recessive mutation cost did not alter the all-mutant population but was associated with a slight decrease in the number and the variability of the homoplasmic-mutant population, whereas a dominant mutation cost markedly decreased both all- and homoplasmic-mutant population size. Simulations also revealed as a general trend the right skewed distribution of mutant parasites per infection (Fig. 1D and Fig. 2). Highly relevant to the outcome of malaria treatment, infections containing mutant parasites before treatment were extremely frequent (Fig. 3): even when considering a conservatively low mtDNA mutation rate of 10^{-10}/nucleotide/replication and parasite burden of $1.2x10^{10}$/body (~0.05% of total host erythrocytes being infected), 100% of simulated

infections contained at least one parasite having at least one mtDNA copy being mutated (corresponding to the 5th cycle in Fig 3A; this proportion decreased to 93% for 10^9 parasites per host). Infections containing at least one homoplasmic-mutant parasite were also frequent: 1.4%, 16.3%, and 88.3% at the 5th, 6th and 7th cycle, respectively. The proportion of patients carrying high loads of mutant parasites was largely altered by the total parasite load per patient (Fig. 3A and 3B).

In summary, whatever the parameter values tested here, all simulations shared three essential results: *1)* the mtDNA resistance mutation could occur *de novo* and propagate in most infected host during the erythrocytic parasite expansion phase before a treatment has been initiated; *2)* each patient harboured a complex reservoir of cryptic mutant parasite populations, with varied frequencies of parasites having varied numbers of their mtDNA copies being mutated ; *3)* a marked inter-patient variation in the size of this cryptic mutant reservoir was found, even for a unique set of parameters; and *4)*, a small fraction of the patients carried a high load of mutant parasites. The very high rate of patients found to carry mtDNA-mutated parasites would suggest a very high rate of AP treatment failure, unless the current AP regimen retains therapeutic efficacy towards mtDNA-mutated parasites, thereby limiting the extent of treatment failure. Therefore we sought for evidences showing how AP-resistant parasites responded to AP therapy.

How do atovaquone-proguanil/cycloguanil-resistant parasites respond to AP treatment: clinical observations. Within the ~6,200 *P. falciparum* imported malaria cases that were treated with AP and reported to the French malaria reference center between 2002 and 2012, we identified 7 cases in which recrudescing AP-resistant infections were treated again with AP (Table 1). Full case reports are described in Supplementary Text. These clinical observations were backed up with the genotyping of atovaquone and cycloguanil resistance markers and of microsatellites to assess the identity of the pre-treatment and post-treatment recrudescing parasites. For each patient, the genetic data showed the selection after the first AP treatment of mutant *cytb*^{268} parasites having a nuclear genetic background identical to the one of parasites from the primary infection. This is consistent with our earlier findings (*11-12*). For five of these patients (patients P1 to P5) the treatment of the AP-resistant infections with another cure of AP was associated with a late failure with parasites recrudescing 8 to 30 days after therapy initiation (mean: 20.6 days), whereas for the other two patients the treatment was altered at day 3 and 5 although their initial parasitemia had significantly decreased. For five cases (P3 to P7), evidence for AP-resistant parasite clearance within days following treatment was obtained from microscopic examination of blood smear, and for three cases (patients P1, P4 and P7) no standard antimalarial drug in addition to atovaquone, proguanil, and cycloguanil was detected in the blood during treatment (these informations were not available for the other cases).

Based on the patients P3, P6, and P7 for which two non-negative and consecutive blood parasite counts were available (at day-0 and day-3 or day-5; table 1), we estimated the parasite reduction ratio for AP-resistant infections (PRR^{APR}) during the first days of AP therapy to be 116, 10, and 30 (mean: 52), respectively. Altogether, these clinical findings showed that some AP-resistant parasites can be

316 eliminated by AP as long as drug blood concentrations are maintained above a yet unknown threshold
317 value, which we defined as the minimum parasiticidal concentration (MPC^{APR}).
318 **Estimation of the minimum time required to clear the cryptic mtDNA-mutated parasites.** We
319 estimated the time (T) during which drug concentrations in the blood should exceed or equal the
320 MPC^{APR} to clear from any host all the cryptic mutant parasites that emerged during the primary
321 infection. Assuming a constant PRR^{APR} of 50, $T = 0.51\ \ln B_m$ (where B_m is the mutant parasite load per
322 patient). In hyperparasitemic patients carrying about $1.8x10^{12}$ parasites (~7% of total host erythrocytes
323 being infected), the maximum load of homoplasmic-mutant parasites found before treatment among
324 20,000 simulations was 176,395 parasites/host (simulation parameters: recessive mutation, mutation
325 rate = 10^{-10}/nucleotide/replication, mtDNA copy number = 20). The MPC^{APR} should then be
326 maintained during at least 6.2 days to ensure the complete elimination of this homoplasmic-mutant
327 intra-host population (Table 2). When considering mutant parasites having at least 1/20 or 10/20 of
328 their mtDNA copies being mutated, drug blood concentrations equal to or larger than the MPC^{APR}
329 should be maintained during a longer length of time to produce similar treatment efficacy. In non
330 hyperparasitemic patients (for example carrying about $1.5x10^{11}$ or $1.2x10^{10}$ parasites which correspond
331 to ~0.6% and ~0.05% of total host erythrocytes being infected), the MPC^{APR} should be maintained for
332 shorter time to clear all the mutant parasites (Table2).
333

DISCUSSION

We report here new findings that help to explain the easy and repeated evolution of mtDNA mutations conferring atovaquone resistance and the high rates of atovaquone and AP treatment failures in non or little immune patients (6, 19, 21, 43). Firstly, our work explores for the first time the intra-host dynamics of malarial mtDNA mutations through stochastic modeling at the micro-evolutionary time scale, id est during few erythrocytic parasite cycles. Computational data show that almost any primary malarial infection in a non-immune human host carries a cryptic population of *de novo* mtDNA-mutated parasites at the time of malaria diagnosis, before a treatment has been initiated. Given the large parasite load per human body at malaria diagnosis (the median peripheral parasitemia of 0.4% in travellers roughly corresponds to $1.2x10^{11}$ parasites per body) and that each parasite contains ~20 copies of mtDNA (24), the intra-host population size of mtDNA molecules grows to extremely large values. This provides multiple opportunities for random mtDNA mutations to occur within each infected host. The extremely low intra-host frequency of these *de novo* mtDNA mutations (~ a median of 1 to 10 times the mutation rate, according to total parasite load per patient, Table S1) precludes their detection in patient blood samples with standard molecular techniques. Indeed, in hyperparasitemic patients carrying ~$1.8x10^{12}$ parasites and assuming 5 liters of blood per patient, the model indicated that a mutant allele is present at an intra-host median frequency of ~10^{-9} (simulation parameters: recessive mutation, mutation rate = 10^{-10}/nucleotide/replication, mtDNA copy number = 20) which translates to ~3 mutant allele copies/10 mL of blood (this increased to 36 allele copies/10 mL of blood in top 1% of patients with high mutant parasite burden). This is consistent with genetic tests that failed to detect the resistance mutation in the pre-treatment blood samples (19-21, 44-45), even using a mutant-enrichment method (22). Highly sensitive methodologies based on next-generation sequencing which can detect one mutant copy among about 10^6-10^7 wild-type copies could be helpful in the future to test our computational results (46-47). In summary, the model findings point to spontaneous mtDNA mutations occurring before treatment as a primary source of atovaquone resistance mutations. And we consider it is not necessary to invoke a mutagenic effect of atovaquone (see Supplementary Text for further discussion).This proposal is in line with findings in *Mycobacterium tuberculosis* reporting the *de novo* acquisition of multidrug resistance before treatment as a risk factor for treatment failure (48), and with mathematical modeling studies in several pathogens (48-51). Secondly, we report novel clinical cases showing that the drug blood concentrations attained during the first days of AP therapy are high enough to clear some AP-resistant parasites. We provide an estimate for the PRR^{APR} (mean: 52 ; see Supplementary Text for discussion associated with this estimate), not markedly different from the values obtained for AP-sensitive infections which ranged from 46 to 261 in non-immune adults (52). The reasons for this finding remain elusive but we propose several hypotheses. At high concentrations, atovaquone can inhibit both wild type and mutant cytochrome *b* (12) and was found also to be a more general ubiquinone antagonist, inhibiting the substrate-ubiquinone reductases of the respiratory chain distinct from the cytochrome *bc1* complex (53). In

addition, it is the net effect on parasite killing of high concentrations of atovaquone, proguanil and cycloguanil in combination that should be considered, an issue raised recently also for chloroquine (54). An estimate for the MPC^{APR} is yet to be determined and the MPC^{APR} will likely be a complex interplay between atovaquone, proguanil and cycloguanil blood concentrations and the threshold number of mutated mtDNA copies per parasite that confers resistance to atovaquone.

Altogether, our computational and clinical results support the following *scenario* regarding *de novo* AP treatment failures. AP treatment success relies in almost any patient on the clearance of cryptic mtDNA-mutated parasites that evolved during the erythrocytic growth phase of the primary infection. Clearance of mutants must occur within the first days of AP treatment, when drug blood concentrations are the highest and exceed the MPC^{APR}. We then refer to the time during which drug blood concentrations exceed or equal the MPC^{APR} as the window of mutant prevention. We suggest that the therapeutic margin of the current AP regimen, especially in the context of non-supervised treatment, is not wide enough to clear all the cryptic mutant parasites in any patient, hence producing repeatedly *de novo* AP treatment failures. The main reasons for this, based on our data and the literature, could be the inter-patient variability of the following parameters: the width of the mutant prevention window and the number of *de novo* mtDNA-mutated parasites that the treatment has to destroy. With regards to the width of the mutant prevention window, a major source of variability is obviously the large inter-patient variability in atovaquone blood concentration which is driven by the slow and limited absorption of the drug (55-56). In addition, this is likely made worse in the context of non-supervised treatment because of potential vomiting and/or poor compliance with the 3-day course of treatment. Another source of variability could be the level of atovaquone resistance conferred by the *cytb*^{268} mutation which will likely alter the MPC^{APR}, hence the width of the mutant prevention window. With regards to the *de novo* mutant parasite load, the model revealed a very large inter-patient variability. This variability was contributed largely by some type of random drift specifically associated with mtDNA replication and partitioning during mitosis and also by the inter-patient variability in total parasite load at diagnosis. As shown also by modeling studies for nuclear mutations (51, 57), the larger is the total parasite load, the larger the *de novo* mutant load, and the longer it will take to eliminate all *de novo* resistant parasites from an infection. This is consistent with our clinical data and those already published (19-21, 45, 58-60), which altogether showed that 17 of 20 patients experiencing *de novo* AP treatment failure presented with parasitemia at diagnosis close to or larger than 0.4% (corresponding to the median peripheral parasitemia observed at the time of *P. falciparum* malaria diagnosis in travellers returning to France and the UK (38, 61); mean parasitemia: 2.4%, median: 1.4%, min: <0.005%, max: 13%; Table S2). Controlled studies will be needed to confirm this observation. Additional sources of mutant load variability could be contributed by the mtDNA mutation rate (48, 62) and mtDNA copy number, but whether those parameters vary substantially between *P. falciparum* clinical isolates remains to be established.

Here, we did not consider mutations occurring earlier during the primary infection, *id est* during the liver phase (63). This would however even increase the mutant load and the probability of a patient to carry mutant parasites. Regarding the physical mechanisms controlling how malaria organelles and their genome replicate and are passed to daughter cells, this remains a largely unexplored area. During one erythrocytic 48hr-generation, the malarial mitochondrion elongates from a single organelle, then branches and ultimately divides to create multiple daughter organelles (27). Our model assumes 5 consecutive rounds of random mtDNA replication/partitioning during one erythrocytic generation, in which each mtDNA copy behave as an independent unit of replication. We argue that this model mimics the effects of both the likely separation of mtDNA molecules within the elongating and branching mitochondrion and the random drift associated with the mtDNA replication (24-25, 27). Variations of this standard model can be envisaged, such as a more panmictic behaviour of mtDNA copies (24), but we do not expect they will produce qualitatively very different results. In its current version, our model is not designed to evaluate the long term evolution of mitochondrial genetic diversity, either within a single-host infection that would last for several weeks or months or within an entire malaria parasite population across multiple rounds of host/vector transmission. This would require to consider back mutation and host-specific immunity and to take into account the large heterogeneity and complexity of parasite transmission (26, 64). Altogether, our model provides, to our knowledge, the first framework to study the intra-host early evolution of malarial mtDNA mutations. It may provide a valuable addition to the current modeling methodologies that rather focus on nuclear mutations. Specifically, it could be helpful to study the evolution of mitochondrial genes whose products may be targeted by new antimalarial drugs, an area of intense research (65-68).

It is currently not clear how many mutant *cytb* copies per parasite are necessary to confer high-grade atovaquone resistance. Our model did not simulate the fate of parasites having distinct mtDNA genotypes (wild type, heteroplasmic, homoplasmic-mutant) once treatment has been initiated, hence under drug selection. Experiences in yeast show that the effect of drug selection will largely overshadow the one of random drift (69). We speculate that once AP treatment has started, intercellular selection within the host will likely favour the growth of those parasites having the largest proportions of their mtDNA copies being mutated. In case some heteroplasmic genotypes could survive to AP treatment, the combination of random drift and strong drug selection would result in a progressive shift in the mutant parasite population, with the progressive accumulation of homoplasmic-mutant parasites (as shown experimentally in yeast (69); see also Supplementary Text).

Our model for the emergence of atovaquone resistance suggests that the partner drug -here proguanil together with its active metabolite cycloguanil- plays a critical role in preventing the escape of *de novo* mtDNA mutant parasites. This is consistent with the observation that most atovaquone resistance mutations reported to date were found to emerge in parasites carrying a mutant *dhfr* gene (Table S2).

Controlled studies with a large patient cohort will be necessary to confirm these observations. Speculating on the computational model findings and the high rate of *de novo* AP treatment failure reported in travellers, we warn against the risk of the rapid emergence of atovaquone resistance if AP is used massively with the current regimen in transmission areas where people are little- or non-immune (2-3). This could be particularly rapid in the multiple areas where mutant *dhfr* gene is found at a high rate in parasite populations (15), as a consequence of a long history of sustained use of the antifolate pyrimethamine.

The framework proposed here suggests several ways by which to delay the clinical emergence of AP resistance. A first strategy could be to extend the window of mutant prevention by maintaining atovaquone and proguanil/cycloguanil blood concentrations at a higher level and for a longer period of time than those achieved with the current AP dosage regimen. The benefit of a more aggressive chemotherapy in reducing the chance of *de novo* resistance selection has been recently questioned (1, 70). However, the clinical findings that AP at high drug blood concentrations remains potent on AP-resistant parasites suggest such a strategy merits further attention. A second (and possibly complementary) strategy that we favour would be to minimize the number of *de novo* mutant parasites that AP has to destroy. This could be achieved through combination therapy, employing an additional drug equally potent against AP-sensitive and AP-resistant parasites. For example, the triple combination of artesunate-atovaquone-proguanil proved to be very efficient for the treatment of malaria in pregnancy and of uncomplicated malaria (71-73). Also, the use of AP could be restricted to treat infections from endemic areas where the frequency of mutant *dhfr* genes is low. Finally, an important prediction of our model is that patients with higher parasite burden at diagnosis face an increased risk for carrying larger *de novo* mutant parasites. Then the use of AP as treatment could be restricted to patients with lower parasitemia at diagnosis.

In summary, we report new computational and clinical findings describing the intra-host evolution of mtDNA mutations conferring atovaquone resistance. The mechanistic framework proposed here helps to explain the repeated evolution of *de novo* AP resistance, and it could help policy makers in designing AP-containing treatment regimens that delay the clinical emergence of atovaquone resistance.

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712 **Figure legends**

713

714 **Figure 1. Dynamics and loads of mtDNA mutant parasites per human host.** Data are from
715 20,000 simulations, with one simulation representing one human infection. ***A***, triangles
716 represent homoplasmic-mutant parasites and circles represent all-mutants (either
717 heteroplasmic or homoplasmic-mutants); plain and dashed lines represent the median and the
718 99.5th percentile, respectively. ***B***, triangle and circle symbols represent homoplasmic-mutant
719 and all-mutants parasites, respectively. ***C*** and ***D***, data are from simulations stopped at the 6th
720 erythrocytic 48h-cycle following parasite delivery from the liver (corresponding to a mean
721 total parasite load per host of $1.5x10^{11}$). The load of mutant parasites per host is shown,
722 according to the number of mtDNA mutant copies per parasite. ***C***, each line (either plain or
723 dashed) represents one simulated infected host randomly chosen among 20,000 simulations.
724 Five simulations are shown. ***D***, box-plots show the median and inter-quartile range (IQR), and
725 the bottom and top whiskers show the lowest data still within 1.5xIQR of the lower quartile
726 and the highest data still within 1.5xIQR of the upper quartile, respectively. Outlier values are
727 shown as dots. There is no mutation cost, the mtDNA copy number is 20, and the mutation
728 rate is 10^{-10}/nucleotide/replication in all the simulations presented here.

729

730 **Figure 2. Load of mtDNA mutant parasites per human host according to different**
731 **parameters.** Data are from 20,000 simulations, with one simulation representing one infected
732 host. **A, C, E and G:** refer to all-mutant parasites, *id est* having at least one of their *n* mtDNA
733 copies being mutated. **B, D, F and H:** refer to homoplasmic-mutant, *id est* parasites having all
734 of their *n* mtDNA copies being mutated. **E** and **F**, at the 5th, 6th and 7th erythrocytic 48hr-
735 cycles, there is a mean number of $1.2x10^{10}$, $1.5x10^{11}$, and $1.8x10^{12}$ parasites per host in our
736 simulations, respectively. Except if differently stated, mutation rate is 10^{-10}/nucleotide/replication, there is no resistance mutation cost (neutral), the mtDNA copy
738 number is 20, and the parasite load per host is $1.5x10^{11}$ (corresponding to the 6th erythrocytic
739 48hr-cycles following parasite delivery from the liver). neu: neutral, rec: recessive, dom:
740 dominant. Box-plots show the median and inter-quartile range (IQR), and the bottom and top
741 whiskers show the lowest data still within 1.5xIQR of the lower quartile and the highest data
742 still within 1.5xIQR of the upper quartile, respectively. Outlier values are shown as dots.

743

744 **Figure 3. Proportion of infections carrying mutant parasites.** Data are from 20,000
745 simulations, with one simulation representing one human infected host. **A**: y-axis scale ranges

from 0 to 100; **B**: same data as in **A** but y-axis scale ranges from 0 to 2.5. Black, red, and blue lines represent infections after 5, 6, and 7 erythrocytic 48hr-cycles, respectively, following parasite release from the liver. This corresponds to a mean of $1.2x10^{10}$, $1.5x10^{11}$, and $1.8x10^{12}$ parasites per host, respectively. The mtDNA copy number is 20, the mutation rate is 10^{-10}/nucleotide/replication, and the mutation cost is recessive in the simulations presented here. ≥1/20 refers to parasites having at least one of their 20 mtDNA copies being mutated (*id est* all-mutant); ≥10/20 refers to parasites having at least 10 of their 20 mtDNA copies being mutated; and 20/20 refers to parasites having all of their 20 mtDNA copies being mutated (*id est* homoplasmic-mutant).

Table 1. Patients reported in the study.

							Plasma drug (μM)[□,**]				Parasite genetics[††]						
													Microsatellites[§§]				
Patient[*]	Sex	Age (year)	Weight (kg)	Isolate (day)[†]	Drug[‡]	Parasitemia (%)	Ato	Pro	Cyc	Others	*cytb*	*dhfr*	TAA 81	TAA 87	PfPk2	Ara2	TAA 60
P1	M	24	55	d0	AP	<0.005	-	-	-	-	Y	-	179	93	-	-	94
				d25/d0	AP	0.1	-	-	-	-	S	IRN	179	93	169	103	94
				d26/d1		-	1.20	0.48	0.18	neg	-	-	-	-	-	-	-
				d45/d20	QN	0.4	-	-	-	-	S	IRN	179	93	169	103	94
P2	M	12	40	d0	AP	2.9	neg	neg	neg	neg	Y	IRN	173	84	172	106	82
				d8		neg	-	-	-	-	-	-	-	-	-	-	-
				d35/d0	AP	0.5	neg	neg	neg	neg	S	IRN	173	84	172	106	82
				d65/d30	MFQ	<0.1	-	-	-	-	S	IRN	173	84	172	106	82
P3	M	9	53	d0	AP	6.6	neg	neg	neg	neg	Y	IRN	182	-	172	103	95
				d7		neg	4.12	0.02	neg	neg	Y	IRN	182	-	172	103	-
				d29/d0	AP	12.5	-	-	-	-	C	IRN	182	-	172	103	95
				d32/d3		0.01	-	-	-	-	C	-	-	-	172	103	95
				d49/d20	QN	3.5	0.39	neg	neg	neg	C	IRN	182	-	172	103	95
P4	F	59	64	d0	AP	1	-	-	-	-	-	-	-	-	-	-	-
				d12		neg	2.39	neg	neg	neg	Y	-	-	-	-	-	-
				d24/d0	AP	1	-	-	-	-	C	IRN	179	81	163	109	95
				d32/d8		neg	7.98	0.04	neg	neg	C	-	-	81	163	-	95
				d46/d22	QN	0.2	2.54	neg	neg	neg	C	IRN	179	81	163	109	94
P5	F	14	52	d0	AP	0.3	-	-	-	-	Y	IRN	179	84	169,172	103	79
				d10		neg	-	-	-	-	-	-	-	-	-	-	-
				d23/d0	AP	0.1	-	-	-	-	S	IRN	-	-	-	-	-
				d31/d8		<0.1[§]	4.10	neg	neg	-	S	IRN	-	-	-	-	-
				d39/d16	MFQ	0.25	-	-	-	-	S	IRN	179	84	172	103	79
P6	M	0.5	8	d0	AP	4	-	-	-	-	-	-	-	-	-	-	-
				d3		<0.1	-	-	-	-	Y	IRN	179	96	190	112	91
				d19/d0	AP	6	-	-	-	-	Y+C	IRN	179	96	190	112	91
				d22/d3	MFQ	0.2	-	-	-	-	-	-	-	-	-	-	-
P7	M	58	70	d0	AP	2	neg	neg	neg	neg	Y	IRN	164	93	166	127	91
				d22		neg	-	-	-	-	-	-	-	-	-	-	-
				d27/d0	AP	1.5	0.10	neg	neg	neg	C+S[‡]	IRN	164	93	166	127	91
				d32/d5	QN	0.0003[¶]	3.62	0.09	0.04	neg	C+S	IRN	164	93	166	127	91

*, patients P1 to P7 were infected in The Union of the Comoros, Mali, Senegal, Gabon, Ivory Coast, The Union of the Comoros, and Togo, respectively. All are of African ethnicity except P7 who is Caucasian; patient P5 was already reported in (11); additional informations and full case report are provided here.

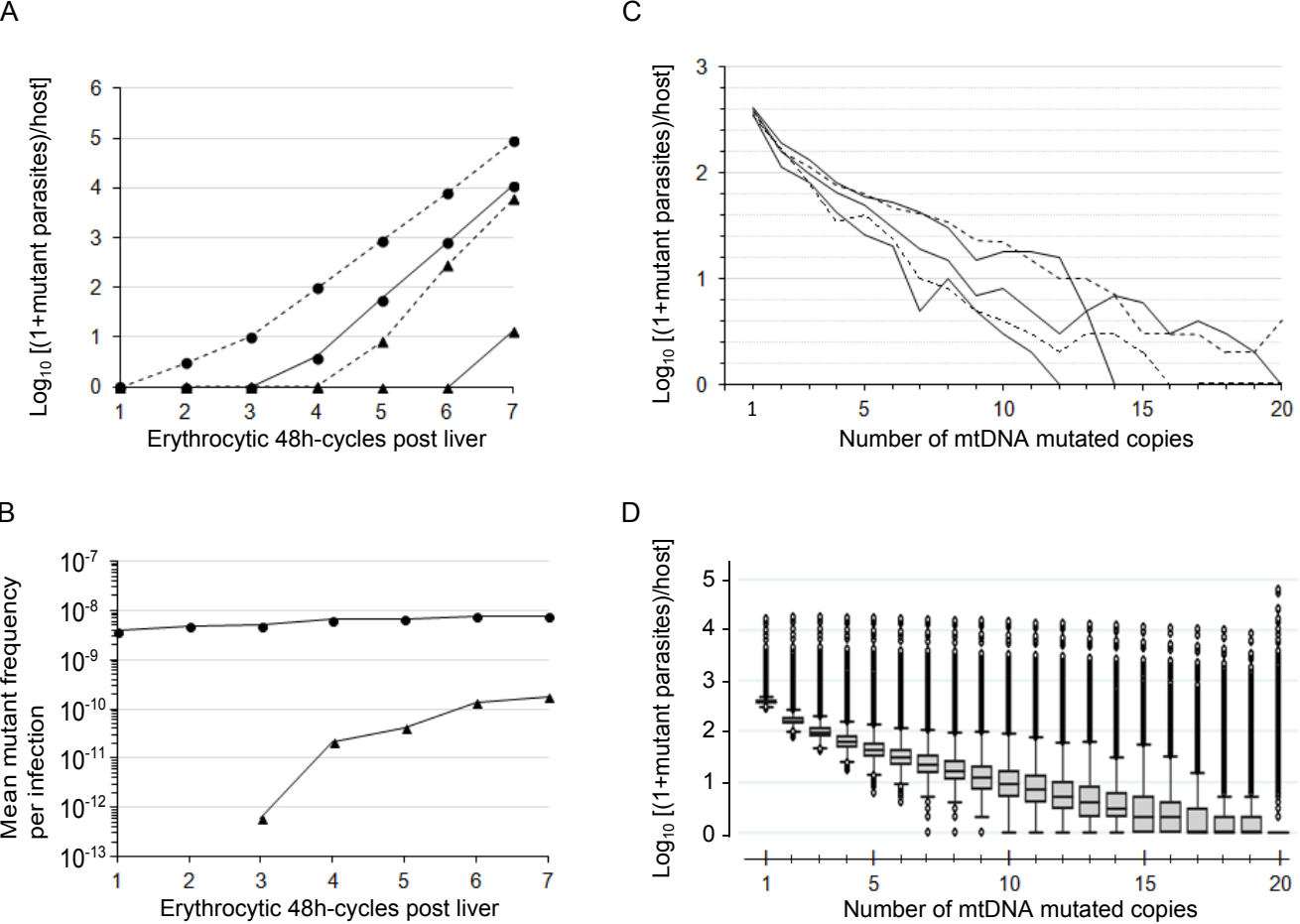
†, d0 corresponds to the day of diagnosis and treatment initiation.

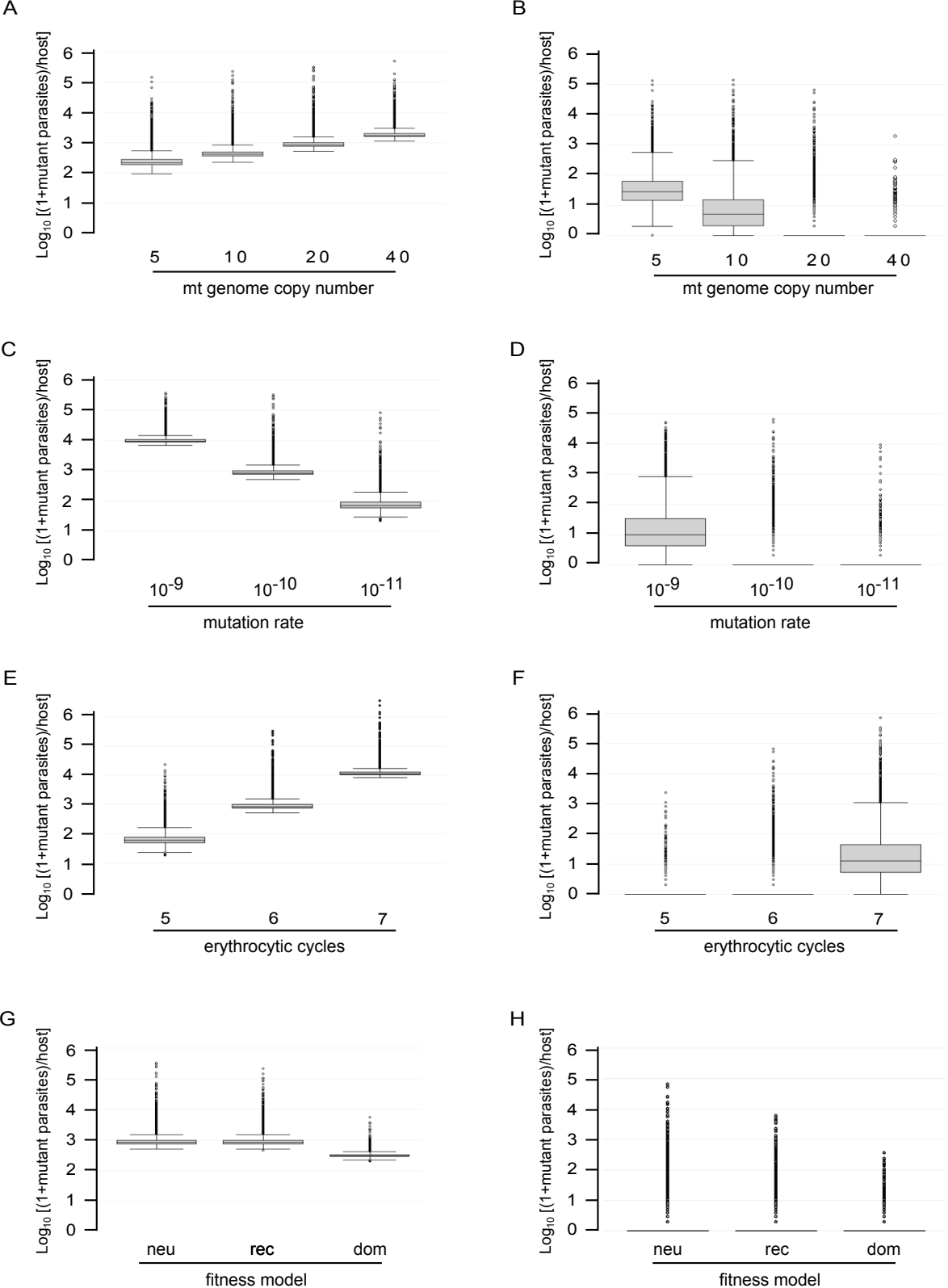
‡, drug used for treatment; AP, atovaquone-proguanil; MFQ, mefloquine; QN, quinine.
§, few trophozoïtes detected on the thin and thick smears.
¶, calculated peripheral parasitemia, based on 14 parasites /µL detected on the thick smear and a measured erythrocyte concentration of 3.7 T/L.
, Ato, atovaquone; Pro, proguanil; Cyc, cycloguanil; Others: monodesethyl-chloroquine, chloroquine, doxycycline, sulfadoxine, pyrimethamine, mefloquine, carboxy-mefloquine, amodiaquine, monodesethyl-amodiaquine, desbutyl-halofantrine, lumefantrine, dihydro-artemisinin; the lower limit of quantification was 5µg/L, which corresponds to 0.014µM, 0.020µM, and 0.020µM, for atovaquone, cycloguanil and proguanil, respectively.
**, "neg" means not detected ; "-" means not done.
††, *cytb*: codon 268 was analyzed, wild type is Tyrosine (Y) and mutants are Serine (S) and Cysteine (C); the mutations *cytb* Y268S and Y268C confer resistance to atovaquone; *dhfr*: the triple *dhfr* mutation N51I+C59R+S108N confers resistance to cycloguanil.
‡‡, the presence of the 2 mutations Y268S and Y268C within the same isolate was confirmed by cloning the PCR product and subsequent sequencing of multiple clones (supplementary figure 1).
§§, allele size (in base pair) of PCR products are reported.

776 **Table 2.** Time required to clear the *de novo* mtDNA mutant parasites emerging from primary
777 infections.
778

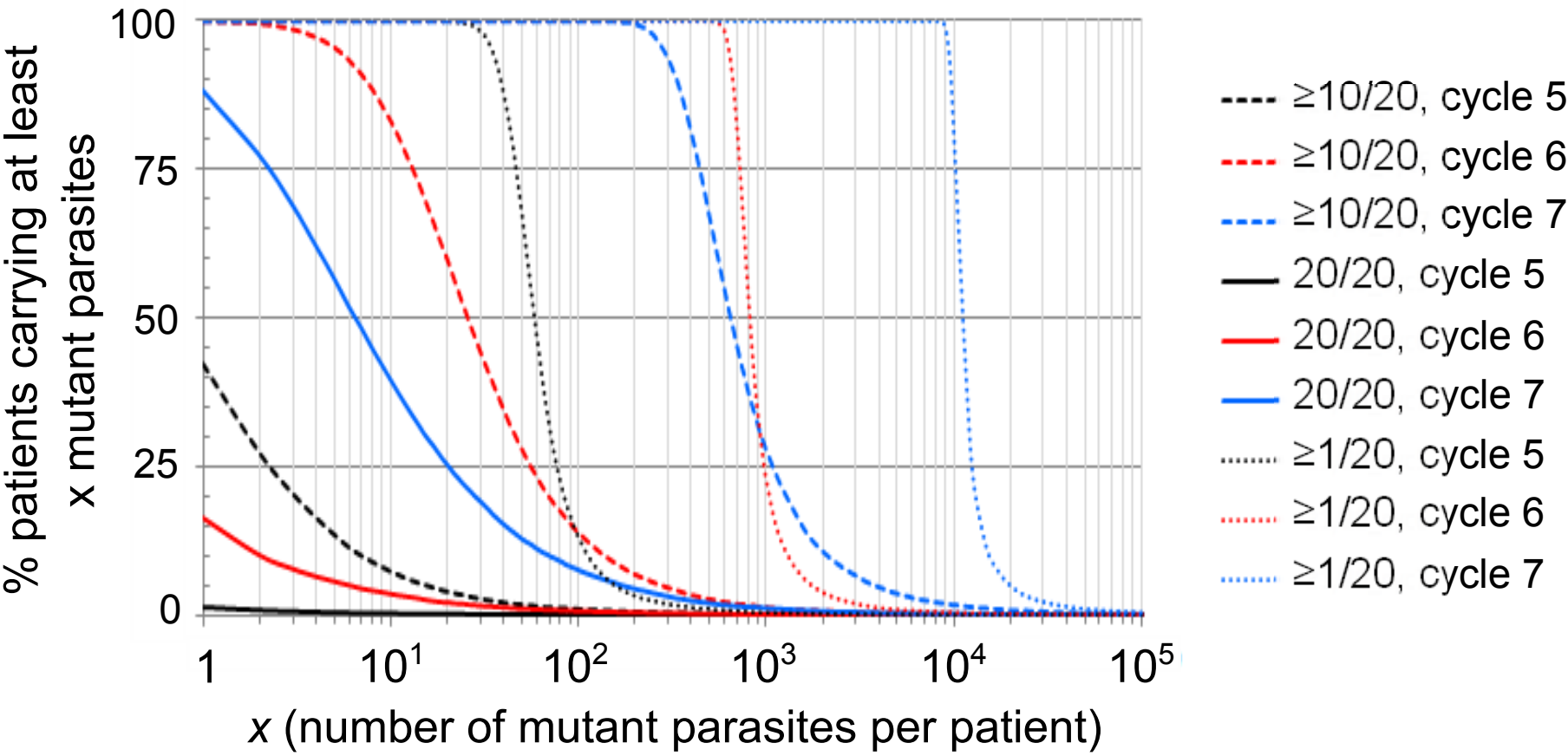
Total parasite load per patient	Fraction of mutated mtDNA copies[¶]	Mutant parasite load per patient[*]			Mutant clearance time (days)[†]
		Median	Min.	Max.	
$1.8x10^{12}$	20/20	6	0	176,395	6.2
	≥10/20	651	99	1,480,258	7.2
	≥1/20	11,132	8,418	3,049,318	7.6
$1.5x10^{11}$	20/20	0	0	6,157	4.5
	≥10/20	25	0	79,843	5.8
	≥1/20	825	443	210,890	6.2
$1.2x10^{10}$	20/20	0	0	448	3.1
	≥10/20	0	0	6,960	4.5
	≥1/20	58	17	18,352	5.0

779
780 [¶], ≥1/20 refers to parasites having at least one of their 20 mtDNA copies being mutated (*id est* all-mutant);
781 ≥10/20 refers to parasites having at least 10 of their 20 mtDNA copies being mutated; and 20/20 refers to
782 parasites having all of their 20 mtDNA copies being mutated (*id est* homoplasmic-mutant)
783 [*], Data are from 20,000 simulations performed with the following parameters: recessive mutation cost, mtDNA
784 copy number = 20, and mutation rate = 10^{-10}/nucleotidic site/replication.
785 [†], Assuming a constant parasite reduction ratio for AP-resistant parasites of 50. Maximum (Max.) mutant
786 parasite loads are used for the estimation.

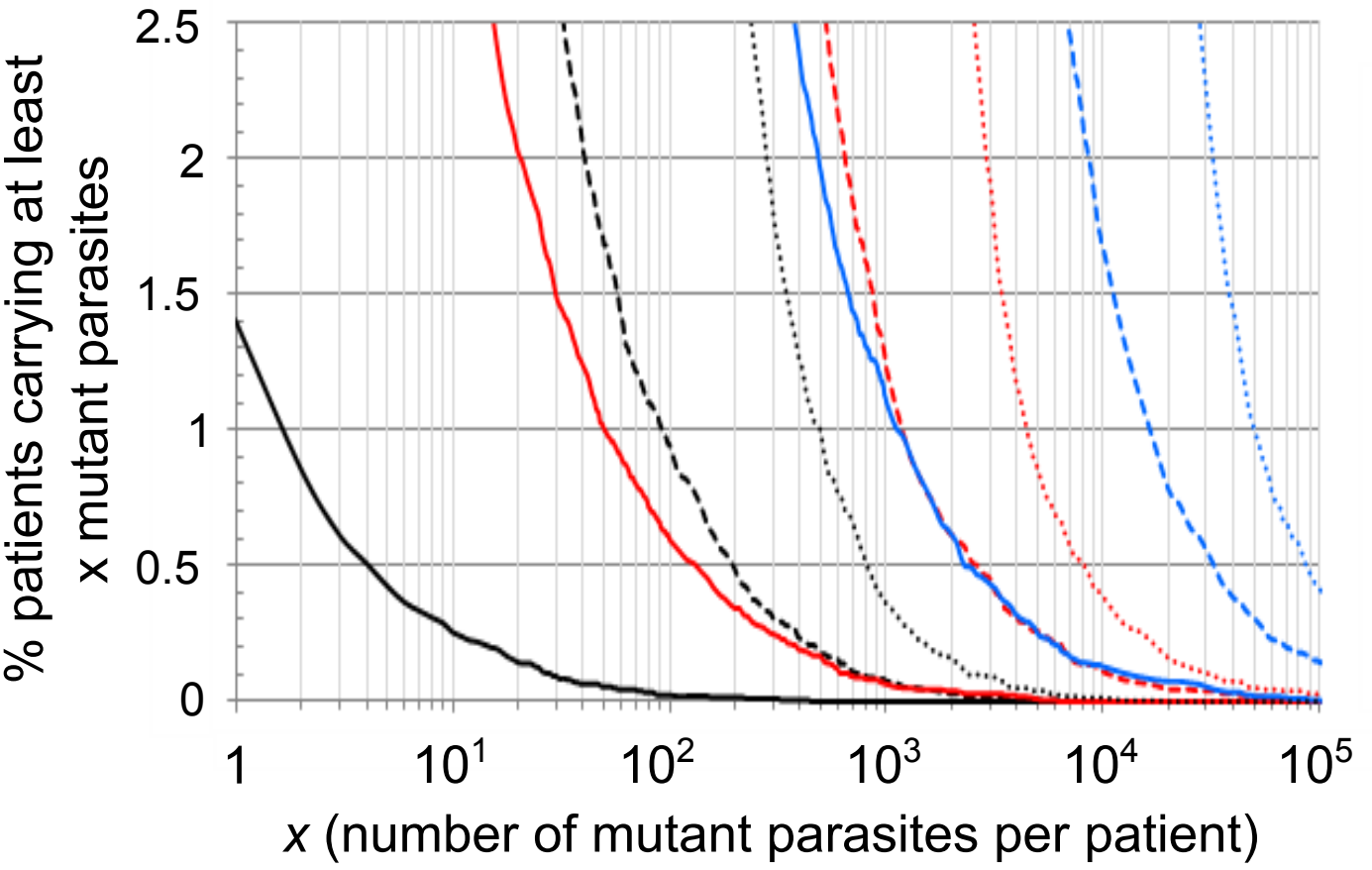

A
Log10 [(1+mutant parasites)/host]
Erythrocytic 48h-cycles post liver
B
Mean mutant frequency per infection
Erythrocytic 48h-cycles post liver
C
Log10 [(1+mutant parasites)/host]
Number of mtDNA mutated copies
D
Log10 [(1+mutant parasites)/host]
Number of mtDNA mutated copies


A
B
C
D
E
F
G
H
Log10 [(1+mutant parasites)/host]
0
1
2
3
4
5
6
5
10
20
40
mt genome copy number
10-9
10-10
10-11
mutation rate
5
6
7
erythrocytic cycles
neu
rec
dom
fitness model

A


% patients carrying at least x mutant parasites
100
75
50
25
0
1
10^1
10^2
10^3
10^4
10^5
x (number of mutant parasites per patient)
≥10/20, cycle 5
≥10/20, cycle 6
≥10/20, cycle 7
20/20, cycle 5
20/20, cycle 6
20/20, cycle 7
≥1/20, cycle 5
≥1/20, cycle 6
≥1/20, cycle 7

B


% patients carrying at least x mutant parasites
2.5
2
1.5
1
0.5
0
1
10^1
10^2
10^3
10^4
10^5
x (number of mutant parasites per patient)

Supplementary materials

1) The mathematical description of the model:

The following sections describe the appearance of mtDNA mutations and their propagation to daughter mtDNA copies and parasites along one erythrocytic 48hr-cycle. This erythrocytic 48hr-cycle was repeated up to 7 times in a row to simulate the parasite blood stage growth during a *P. falciparum* malaria primary infection in a non-immune patient. During one intra-erythrocytic 48h-cycle, each *P. falciparum* parasite undergoes 5 consecutive endo-mitosis (that occur within a single parasite), resulting theoretically in the production of $2^5 = 32$ daughter parasites per initial parasite.

Spontaneous mutation

At the mtDNA replication d, each wild type parasite has a probability of spontaneous mtDNA mutation $p = 1 - (1 - \mu)^n$, where μ is the mutation rate and n is the mtDNA copy number.

The probability of 2 or more spontaneous mutations in the same parasite as well as the probability of a spontaneous mutation among the parasite populations $P_{1,d}$, …, $P_{n,d}$ were assumed to be negligible (where $P_{i,d}$ is the population of mutant parasites having i mtDNA copies being mutated).

At each mtDNA replication d, the number of wild type parasites which mutate spontaneously has a binomial distribution $Bin(N_{0,d}, p)$, where $N_{0,d}$ is the number of wild type parasite at the division d.

Duplication of the mtDNA

The n mtDNA template copies to be duplicated during one mtDNA replication are randomly drawn with replacement among the n initial mtDNA copies. Thus, for a parasite with k mutated mtDNA copies (k in {0, …, n}), the number M of mutated mtDNA copies that are duplicated during one division has a binomial distribution Bin(n, k/n), with the corresponding probability $p\_repl_i = P(M=i)$.

The $N_{k,d}$ parasites with k mtDNA mutated copies at replication d generate potentially (at most) n+1 distinct mutant parasite populations having a duplicated mtDNA genome: the population $P_{k,d,0}$ for which 0 mutated mtDNA copy have been duplicated, the population $P_{k,d,1}$ for which 1 mutated mtDNA copy have been duplicated…, the population $P_{k,d,n}$ for which n mutated mtDNA copies have been duplicated.

All the parasites of a population $P_{k,d,x}$ have k+x mutated mtDNA copies among 2n (x in {0, …, n}). ($N_{k,d,0}$, $N_{k,d,1,\ldots,}N_{k,d,n}$) has a multinomial distribution $M(N_{k,d}, p\_repl_0, p\_repl_1, ..., p\_repl_n)$, where $N_{k,d,x}$ is the size of a parasite population $P_{k,d,x}$ and $\sum_{x=0}^{n} N_{k,d,x} = N_{k,d}$

Partitioning of the mtDNA copies among 2 daughter separated mitochondrial units

The mtDNA duplication for all parasite populations $P_{1,d}$, …, $P_{n,d}$ at the replication d generates potentially 2n+1 parasite populations $P'_{k,d}$ (k in {0, …, 2n}), where k is the number of mutated mtDNA copies among 2n. The segregation of n mtDNA copies is modelled as the random draw without replacement of n mtDNA copies among 2n, generating 2 separated daughter mtDNA unit

having each n mtDNA copies. The daughter mtDNA units being separated, they were further considered as independent daughter "transient" parasites for convenience.

First case: k<n

Each parasite of a population $P'_{k,d}$ generates one of the k+1 different couples C^1_j of daughter parasites (j in {0, …,k}), with a probability $P_{C^1_j}$ according to an hypergeometric distribution H (2n, k, n). A couple C^1_j contains a daughter parasite with j mutated mtDNA copies among n and the other with k-j mutated mtDNA copies among n.

The $N'_{k,d}$ parasites of the population $P'_{k,d}$ generate $N'_{k,d,j}$ couples C^1_j, and $\sum_{j=0}^{k} N'_{k,d,j} = N'_{k,d}$

($N'_{k,d,0}$, $N'_{k,d,1,\ldots,}N'_{k,d,k}$) has a multinomial distribution M($N'_{k,d}$, $P_{C^1_0}$, $P_{C^1_1}$,…, $P_{C^1_k}$).

Second case: k ≥ n

Each parasite of a population $P'_{k,d}$ generates one of the k+1 different couples C^2_j of daughter parasites (j in {0, …,k}), with a probability $P_{C^2_j}$ according to an hypergeometric distribution H (2n, k, n). A couple C^2_j contains one daughter parasite with j wild type mtDNA copies among n and the other with k-j wild type mtDNA copies among n.

The $N'_{k,d}$ parasites of the population $P'_{k,d}$ generate $N'_{k,d,j}$ couples C^2_j and $\sum_{j=0}^{k} N'_{k,d,j} = N'_{k,d}$

($N'_{k,d,0}$, $N'_{k,d,1,\ldots,}N'_{k,d,k}$) has a multinomial distribution M($N'_{k,d}$, $P_{C^2_0}$, $P_{C^2_1}$,…, $P_{C^2_k}$)

After 5 consecutive cycles of this duplication-partioning process (which mimic the 5 consecutive endo-mitosis that occur within a single parasite during its erythrocytic 48h-cycle), the number of survival parasites from each genotypically distinct parasite population (wild-type, heteroplasmic, homoplasmic-mutant) is drawn in a binomial distribution Bin(Ni, p_surv), where Ni is the number of parasite of the parasite population with i mutated copies (from 0 to n) and p_surv is the survival probability of each parasite population (varying according to the number of mutated mtDNA copies per parasite, as explained in the main section). Each surviving parasite will initiate a new erythrocytic 48hr-cycle.

2) Supplementary results & discussion

<u>Case reports</u>:

Patient 1 is a 24-year-old African male (weight: 55 kg) living in La Réunion Island, France. He visited family during three months in an urban area in The Union of the Comoros in 2008-09. He declared not taking any antimalarial chemoprophylaxis. Thirteen days after returning home he started experiencing fever and vomiting. A peripheral blood film examination performed at the hospital

laboratory showed *P. falciparum* (parasitemia: <0.005%). A standard 3-day AP treatment (1,000 mg of atovaquone and 400 mg of proguanil, once a day for 3 days) was prescribed but was incompletely taken: the patient received the first dose of AP at the hospital and went home. He took the second dose at home while vomiting, and did not take the third dose because he felt better. At day-25 the patient presented to the hospital and blood smears showed *P. falciparum* parasites (parasitemia: 0.1%). The patient was admitted to hospital for 4 days, and with the history of vomiting during the treatment of the first malaria episode, the recrudescent infection was treated again with a standard 3-day AP treatment. No vomiting was reported during this treatment period. At day-45 (day-20 after the treatment of the recrudescent infection) the patient presented to the hospital with fever (symptoms have started 4 days earlier) and blood smears showed *P. falciparum* parasites (parasitemia: 0.4%). He was admitted to the hospital (4-day stay) and the second recrudescent infection was successfully treated with quinine for seven days. Parasite DNA was extracted from day-0, day-25, day-26 and day-45 isolates (either directly from blood samples or from blood smears or plasma).

Patient 2 is a 12-year-old male weighing 40 kg. He was born in France to African parents living in France. He travelled during 5 weeks to Mali during summer 2010. He declared using chloroquine as antimalarial chemoprophylaxis but stopped prematurely. Eight days after returning to France he started experiencing fever without vomiting. A peripheral blood film examination showed *P. falciparum* parasites (parasitemia: 2.9%). The patient was admitted to the hospital for 2 days and treated with 750 mg of atovaquone and 300 mg of proguanil, once a day for 3 days. Vomiting occurred 1h20min after intake of the first dose. At the day-8 follow-up, no parasites were detected. At day-35, the patient presented to the hospital with fever that started 4 days earlier, headache and body aches ; *P. falciparum* trophozoites were detected on blood film (parasitemia: 0.5%). The patient was treated with 1,000 mg of atovaquone and 400 mg of proguanil, once a day for 5 days instead of 3 days. The patient was not admitted to hospital and the treatment was not supervised. At day-65 (day-30 after the treatment of the recrudescent infection), the patient had fever and trophozoïtes and gametocytes were detected (parasitemia: <0.1%). The second recrudescent infection was successfully treated with oral mefloquine. Parasite DNA was extracted from day-0, day-35, and day-65 isolates (directly from blood samples).

Patient 3 is a 9-year-old African boy (weight: 53 kg) living in France who travelled during two months to Senegal in summer 2011 without antimalarial chemoprophylaxis. Two weeks after returning to France he started experiencing fever and vomiting. A peripheral blood film examination performed at the medical care unit showed *P. falciparum* (parasitemia: 6.6%). The patient was admitted to the hospital (4-day stay) and treated with 1,000 mg of atovaquone and 400 mg of proguanil, once a day for 3 days. Drug intakes (crushed tablets during the meal together with milk and plain yoghurt) were supervised. Vomiting has been reported during hospitalization. Blood film examination at day-3 post-treatment showed parasites (parasitemia: 1.2%). At the day-7 follow-up, both blood film and thick smear showed gametocytes only. At day-29 the patient presented to the hospital with fever and blood

smears showed *P. falciparum* parasites (parasitemia: 12.5%). He was admitted to the hospital (4-day stay) and as vomiting had been reported following the initial treatment he was prescribed another 3-day full course of AP. Drug intakes (crushed tablets during the meal together with milk and plain yoghurt) were supervised. During the 3-day treatment schedule, the course of the treatment was altered because of vomiting events: the patient received 5 doses instead of 3 doses of 1,000 mg of atovaquone and 400 mg of proguanil because two of them were followed by vomiting. At day-32 (day-3 follow-up after the treatment of the recrudescent infection), blood film showed trophozoites (parasitemia: 0.01%). At day-49 (day-20 after the treatment of the recrudescent infection), the patient had fever and trophozoites were detected (parasitemia: 3.5%). He was admitted to the hospital and the recrudescent infection was successfully treated with oral quinine for seven days. Parasite DNA was extracted from day-0, day-7, day-24, day-29, day-32 and day-49 isolates (either directly from blood samples or from blood smears).

Patient 4 is a 59-year-old female weighing 64 kg. She is of African origin (native from Gabon). She is living in France since 25 years and visits Gabon every year. She travelled during 3 weeks to urban an area in Gabon during January/February 2012 without taking antimalarial prophylaxis. Twelve days after returning to France, she experienced a malaria episode with *Plasmodium* parasites evidenced on blood film examination (parasitemia: 1%) and she was treated with a full course of AP (no diet at the first dose). At day-12, no parasites were detected neither using microscopic examination nor PCR typing of peripheral blood. At day-24, the patient presented at the hospital and trophozoites were detected (parasitemia: 1%). The patient was treated again with 1,000 mg of atovaquone and 400 mg of proguanil, once a day for 3 days. At day-32 (day-8 after the treatment of the recrudescent infection), blood film and thick smear were performed and no replicating parasites were detected but gametocytes were observed. At day-46 (day-22 after the treatment of the recrudescent infection), trophozoites were detected (parasitemia: 0.2%). The second recrudescent infection was successfully treated with oral quinine for seven days. Parasite DNA was extracted from day-12, day-24, day-32 and day-46 isolates (directly from blood samples).

Patient 5 is a 14-year-old African girl (weight: 52 kg) living in France, who travelled during one month to Ivory Coast in 2005. She declared using chloroquine as antimalarial chemoprophylaxis. Three days after returning to France she started experiencing fever with abdominal, articular and cervical pain, but without vomiting nor diarrhoea nor obnubilation. A peripheral blood film examination showed *P. falciparum* parasites (parasitemia: 0.3%). The patient was admitted to the hospital for 3 days and was treated with 1,000 mg of atovaquone and 400 mg of proguanil, once a day for 3 days. The treatment however was not supervised and was later reported to be incompletely taken because of vomiting coincidently with 2 of the 3 doses. At the day-10 follow-up, no parasites were detected using microscopic examination of peripheral blood (thin & thick). At day-23 the patient presented to the hospital with fever and vomiting which started 4 days earlier, and headaches. Blood smears showed *P. falciparum* parasites (parasitemia: 0.1%). An acute primary infection caused by

hepatitis A virus was also diagnosed concomitantly. The patient was admitted to the hospital for 4 days. With the history of vomiting during the treatment of the first malaria episode, the recrudescent infection was treated again with a standard 3-day AP treatment. Drug intakes (together with fatty food: milk and plain yoghurt) were supervised and no vomiting were observed. Symptoms disappeared during treatment. At day-31 (day-8 after the treatment of the recrudescent infection), examination of peripheral blood showed many gametocytes and few trophozoites on the thin and thick smear (parasitemia: <0.1%). The family of the patient was contacted. The patient presented to hospital on day-39: she was asymptomatic but trophozoites were detected on blood film (parasitemia: 0.25%). The patient was admitted to the hospital for 3 days and the second recrudescent infection was successfully treated with oral mefloquine (no parasites detected at days 2, 10 and 28 following mefloquine). Parasite DNA was extracted from day-0, day-23 day-31, and day-39 isolates (either directly from blood samples or from blood smears).

Patient 6 is a 5-month-old African boy (weight: 8 kg) living in France, who travelled during two weeks to the Union of the Comoros in 2008 without taking antimalarial prophylaxis. One day after returning to France the family took him to the hospital because of fever, diarrhoea and vomiting which started three days prior. A peripheral blood film examination showed *P. falciparum* parasites (parasitemia: 4%). The patient was admitted to the hospital for 3 days and was treated with 125 mg of atovaquone and 50 mg of proguanil, once a day for 3 days. The treatment was supervised. At the day-3 follow-up, few trophozoites were detected using microscopic examination of peripheral blood (parasitemia <0.1%). At day-19 his mother took him to the hospital because of diarrhoea and fever which started on the same morning. Blood smears showed *P. falciparum* parasites (parasitemia: 6%). The patient was admitted to the hospital for 3 days and was treated with 125 mg of atovaquone and 50 mg of proguanil, once a day for 3 days. Drug intakes (crushed tablets during the meal together with milk and plain yoghurt) were supervised. At day-22 (day-3 after the treatment of the recrudescent infection), trophozoites were detected using microscopic examination of peripheral blood (parasitemia: 0.2%). A treatment with mefloquine was started, and day-26 follow-up showed gametocytes only. Parasite DNA was extracted from day-3 and day-19 isolates (directly from blood samples).

Patient 7 is a 58-year-old male (weight: 70 kg) living in France, who experienced several *P. falciparum* malaria episodes during the last nineteen years. In 2010 he travelled during four days to Togo without taking antimalarial prophylaxis. Seven days after returning home he went to the hospital because of fever and chills without vomiting. A peripheral blood film examination performed at the hospital laboratory showed *P. falciparum* parasites (parasitemia: 2%). A standard 3-day treatment of AP was prescribed and the patient went home. At day-27 the patient presented to the hospital because of with fever and vomiting since 48 hours. Blood smears showed *P. falciparum* parasites (parasitemia: 1.5%). A standard 3-day treatment of AP was prescribed and the patient went home. At day-32 (day-5 after the treatment of the recrudescent infection), few trophozoites were detected using microscopic examination of peripheral blood (calculated parasitemia of 0.0003%, based on 13 parasites /µL

detected on the thick smear and a measured erythrocyte concentration of 3.7 Tera/L). The patient was successfully treated with quinine during 7 days (no trophozoites detected at days 5, 13 and 28 following quinine). Parasite DNA was extracted from day-0, day-27, and day-32 isolates directly from blood samples.

We are not aware of any other AP-resistant infections, as evidenced by molecular analysis and treatment history, which have been treated with AP. However, the pattern of recrudescent infections following two consecutive AP treatments reported in one patient by Kuhn et al. (2005) strikingly resembles the pattern of our 7 cases reports. Unfortunately, some key genetics data were not available for this case, precluding further interpretation regarding how AP-resistant infections respond to AP. Surprisingly, we observed that a significant fraction of AP-resistant parasites were killed by AP during the first days of treatment, leading to a late treatment failure. This is a striking example as the *cytb*^{268} mutation is the canonical single-step mutation that confers high-grade resistance to an antimalarial drug (atovaquone).
These observations could be explained by several factors, as suggested in the main discussion section. In addition, confounding factors could also have contributed to the decrease in parasitemia: *1)* developing acquired immunity (not consistent with the sharp decrease observed concomitantly to treatment administration, and not consistent with similar time to recrudescence and parasitemia for the first and second failure) ; *2)* concurrent intake of other compounds having antimalarial activity ; for 3 of the patients for whom relevant plasma were available (patients P1, P4 and P7), the drug dosage did not detect any additional standard antimalarial ; *3)* residual drug concentrations from the first AP treatment might have resulted in higher drug concentrations when starting the second AP treatment; when available, drug dosages did not show any unusually high plasma drug concentrations.
We estimated the PRR for AP-resistant parasites to be ~50 using microscopy-derived parasite counts from 3 patients. However, caution should be taken regarding the PRR estimate we provide because *1)* one patient (P3) received an altered course of AP (5 doses instead of 3 during the 3-day course of the treatment because two of them were followed by vomiting) and we cannot exclude that drug concentrations attained unusually high values, and *2)* only two values of parasitemias and including day-0 (at day-0 and day-3 for patients P3 and P6, and at day-0 and day-5 for patient P7) were used to estimate the PRR and we cannot assess whether these points were in the linear portion of the parasite clearance curve nor could we take into account the lag phase (Flegg 2011, Nkhoma 2013).

<u>On the origin of the mtDNA mutations conferring atovaquone resistance</u>

1. On the atovaquone-induced mtDNA mutation hypothesis

A popular hypothesis suggests that atovaquone could have a local mitochondrial mutagenic activity through the accumulation of electrons and superoxide, as a consequence of atovaquone blocking the mitochondrial electron transport chain (Srivastava 1999). This could theoretically result in increased

frequency of resistance acquisition. One major drawback with this hypothesis is that more than one mutated copy per parasite would have to be induced, since one mutated copy per parasite is unlikely to confer high-grade atovaquone resistance. Even when considering a massive and transient burst in mtDNA mutation rate, it is very unlikely that several copies of the same parasite will be mutated simultaneously at the *cytb*^{268} codon. Then, propagation of the atovaquone-induced mutation to daughter copies and parasites would require extensive mtDNA replication and cell division which is unlikely to occur during treatment because the high drug concentrations will likely arrest the growth of (and probably destroy) parasites carrying one or few mutated mtDNA copies. Our simulations show that asexual clonal expansion of parasites during the erythrocytic growth phase generates enough mtDNA mutations to repeatedly produce mtDNA mutant parasites before treatment initiation.
In conclusion, we did not test directly the possibility of atovaquone-induced mutations. However we speculate that, if it occurred, atovaquone-induced mutagenesis would contribute marginally to the pool of mtDNA-mutated parasites produced before drug selection.

2. On additional notable findings

First, in one patient (P7) the presence of the 2 different mutations *cytb*^{Y268S} and *cytb*^{Y268C} within the day-of-failure isolates was detected (which was confirmed by cloning and sequencing of the PCR product; supplementary figure 1). This shows that at least two different mutational events occurred at the *cytb*^{268} codon within the same host. These data confirm that mtDNA mutations can be repeatedly produced within short-term single-host infections, which is consistent with our simulations.
Second, in patient P6 the presence of the wild type genotype (*cytb*^{Y268}) was detected in addition to the mutant *cytb*^{Y268C} within the day-of-failure isolate. We have not yet further explored the genotypic description of this complex genotype. We predict that the presence of the wild type allele is contributed by heteroplasmic parasites (having some but not all of their mtDNA copies not mutated) rather that by wild type parasites that would have survived to AP treatment. Experiments have been designed to check this prediction.

Table S1. Distribution of mutant parasites and mutant alleles within infections.

			Percentiles				
	Mean	Median	5th	25th	75th	95th	99th
Recessive, 20 cop, 4 cycles							
Total parasite load	$1.04x10^{9}$	$1.04x10^{9}$					
≥1/20-mutant load	6	3	0	2	6	16	46
≥10/20-mutant load	0	0	0	0	0	0	4
20/20-mutant load	0	0	0	0	0	0	0
Mutation freq (x 10^{-10})	9.1	2.4	0	1.0	5.3	24	100
Recessive, 20 cop, 5 cycles							
Total parasite load	$1.24x10^{10}$	$1.24x10^{10}$					
≥1/20-mutant load	83	58	34	46	77	156	499
≥10/20-mutant load	7	0	0	0	2	15	92
20/20-mutant load	0	0	0	0	0	0	1
Mutation freq (x 10^{-10})	11.8	5.1	2.2	3.5	8.2	5.7	109
Recessive, 20 cop, 6 cycles							
Total parasite load	$1.49x10^{11}$	$1.49x10^{11}$					
≥1/20-mutant load	1,086	825	638	731	984	1,762	4,467
≥10/20-mutant load	117	25	5	13	55	275	1,183
20/20-mutant load	5	0	0	0	0	5	50
Mutation freq (x 10^{-10})	14.4	7.5	4.6	5.9	10.6	28.7	98
Recessive, 20 cop, 7 cycles							
Total parasite load	$1.79x10^{12}$	$1.79x10^{12}$					
≥1/20-mutant load	13,820	11,132	9,497	10,287	12,629	20,080	49,580
≥10/20-mutant load	1,786	651	287	447	1,089	3,778	16,038
20/20-mutant load	102	6	0	2	20	168	1,150
Mutation freq (x 10^{-10})	16.7	9.9	7.1	8.4	12.3	29.5	101

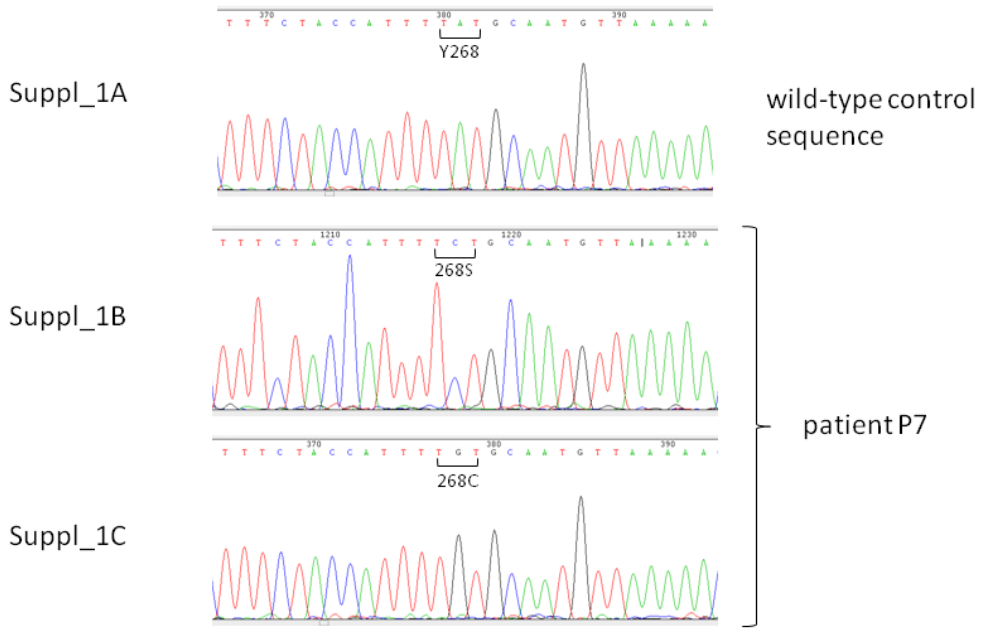
Data are from 20,000 simulations performed with the following parameters: recessive mutation cost, mtDNA copy number = 20, and mutation rate = 10^{-10}/nucleotidic site/replication. ≥1/20 refers to parasites having at least one of their 20 mtDNA copies being mutated (*id est* all-mutant); ≥10/20 refers to parasites having at least 10 of their 20 mtDNA copies being mutated; and 20/20 refers to parasites having all of their 20 mtDNA copies being mutated (*id est* homoplasmic-mutant). The mutation frequency (freq) was calculated as the number of parasite mtDNA mutant copies per host divided by the total number of parasite mtDNA copies per host.

Table S2. Data on 26 published cases of failure of atovaquone-proguanil treatment for *P. falciparum* malaria associated with the *cytb*^{268} mutation.

Age (year)	Country of infection	Parasitemia (%)			*cytb*$^{268*,\$}$		*dhfr*$^{*,\$}$	
		d0	d_{fail}	d_{fail}	d0	d_{fail}	d_{fail}	Ref
38	Burkina Faso	0.35	0.47	22	Y	C	3M	Musset 2006
51	Burkina Faso	nd	0.04	25	nd	S	3M	Musset 2006
36	Guinea	13	5	26	Y	S	3M	Musset 2006
55	Ivory Coast	4	5	26	Y	C	3M	Musset 2006
23	Burkina Faso/Senegal	2.8	1.5	28	Y	S	3M	Musset 2006
18	Comoros	0.5	1.3	23	Y	S	3M	Savini 2008
nd	French Guiana	nd	1	49	Y	S	3M	Legrand 2007
3.5	Mozambique	1.2	3.2	28	nd	S	nd	Rose 2008
21	India	3.4	2	34	nd	C	nd	Perry 2009
29	Sierra Leone	1.1	nd	42	nd	C	nd	Sutherland 2008
5	Nigeria	0.1	0.3	25	nd	S	nd	Sutherland 2008
30	Uganda	nd	0.2	21	nd	C	nd	Sutherland 2008
36	Uganda/Kenya	0.1	<0.1	26	Y	C	nd	Sutherland 2008
47	The Gambia	nd	3	32	nd	C	nd	Sutherland 2008
28	Cameroon	1	2.5	21	nd	S	nd	David 2003
24	Kenya	3	nd	30	Y	S	mut	Schwartz 2003
25	Sierra Leone	nd	nd	19	Y	S	2M	Kuhn 2005
45	Nigeria	1.5	<1	28	nd	N	nd	Fivelman 2002
28	Mali	nd	1.5	28	nd	S	nd	Winchmann 2004
24	Comoros	0.005	0.1	25	Y	S	3M	Current study
12	Mali	2.9	0.5	35	Y	S	3M	Current study
9	Senegal	6.6	12.5	29	Y	C	3M	Current study
59	Gabon	1	1	24	Y	C	3M	Current study
14	Ivory Coast	0.3	0.1	39	Y	S	3M	Current study
0.5	Comoros	4	6	19	Y	Y+C	3M	Current study
58	Togo	2	1.5	27	Y	C+S	3M	Current study

*, d0 and dfail refer to day of diagnosis and day of failure, respectively;
$, The mutations *cytb* Y268S and Y268C confer resistance to atovaquone; 2M and 3M refer to double and the triple *dhfr* mutations, respectively, that confer resistance to cycloguanil.

Supplementary figure 1. Presence of the 2 *cytb* mutations Y268S and Y268C within the day-22 isolate from patient P7. Sequences shown in Suppl_1B and suppl_1C were obtained from different clones of the same PCR product.



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