AMERICAN SOCIETY FOR MICROBIOLOGY | Antimicrobial Agents and Chemotherapy®

CLINICAL THERAPEUTICS

Plasmodium falciparum Recrudescence Two Years after Treatment of an Uncomplicated Infection without Return to an Area Where Malaria Is Endemic

Denis Malvy,[a] Marylin Torrentino-Madamet,[b,c,d] Coralie L'Ollivier,[e,f] Marie-Catherine Receveur,[a] Fakhri Jeddi,[e,f] Laurence Delhaes,[g] Renaud Piarroux,[e,f] Pascal Millet,[g] Bruno Pradines[b,c,d]

[a]Service des Maladies Infectieuses et Tropicales, Hôpital Pellegrin, Bordeaux, France

[b]Unité Parasitologie et Entomologie, Département des Maladies Infectieuses, Institut de Recherche Biomédicale des Armées, Marseille, France

[c]Aix-Marseille Université, Unité de Recherche sur les Maladies Infectieuses et Tropicales Emergentes, UM 63, CNRS 7278, IRD 198, INSERM 1095, Institut Hospitalo-Universitaire (IHU) Méditerranée Infection, Marseille, France

[d]Centre National de Référence du Paludisme, Marseille, France

[e]Laboratoire de Parasitologie, Hôpital la Timone, Marseille, France

[f]Aix Marseille Université, Unité Mixte de Recherche MD3, Marseille, France

[g]Laboratoire de Parasitologie -Mycologie, Hôpital Pellegrin, CHU de Bordeaux, Bordeaux, France

ABSTRACT We report evidence, confirmed by the lack of travel activity outside of France and genetic diversity analysis using polymorphic microsatellite markers, that *Plasmodium falciparum* malaria infection effectively treated with an artemisinin-based combination can remain dormant and relapse during pregnancy at least 2 years after treatment.

KEYWORDS malaria, *Plasmodium falciparum*, antimalarial drug, resistance, *in vitro*, *in vivo*, molecular marker

In 2015, 4,750 imported malaria cases were reported in France, with a mean of 6 days between the end of travel to an area where malaria is endemic and diagnosis (Centre National de Référence du Paludisme, personal communication, B. Pradines). Declaration of symptoms associated with *Plasmodium falciparum* generally does not occur beyond 2 months after returning from a country where malaria is endemic (1). However, some cases of late detection of *P. falciparum* infections have been reported for up to 9 years (2). We describe a molecular-documented case of *P. falciparum* recrudescence 2 years after an uncomplicated infection was first treated.

A woman in her 30s was admitted with febrile presentation to an emergency unit in Bordeaux, France, in 2014. Originally from Cameroon, she moved to France during her childhood and returned to her native country for 10 days in 2014, returning to France 2 weeks before hospital admission. She was under mefloquine prophylaxis during her stay but did not use appropriate protective measures against mosquito bites. She complained of abdominal pain, followed by fever (39°C), headache, and vomiting. She was diagnosed by microscopy with *P. falciparum* (1.6% parasitemia), hospitalized in the infectious diseases department, and treated with dihydroartemisinin-piperaquine 320 mg/40 mg for 3 days (Eurartesim). Drug intake was monitored by nurses, and the routine parasitemia controls by blood smear showed no parasites at days 3, 7, 14, and 21 after treatment. She was examined 9 and 30 days after admission and had no complaints or signs of any disease.

In 2016, the woman was admitted at 24 weeks of pregnancy to the gynecology department of the same hospital in Bordeaux, presenting with fever, vomiting, and

Received 17 September 2017 **Returned for modification** 29 October 2017 **Accepted** 3 December 2017

Accepted manuscript posted online 11 December 2017

Citation Malvy D, Torrentino-Madamet M, L'Ollivier C, Receveur MC, Jeddi F, Delhaes L, Piarroux R, Millet P, Pradines B. 2018. *Plasmodium falciparum* recrudescence two years after treatment of an uncomplicated infection without return to an area where malaria is endemic. Antimicrob Agents Chemother 62:e01892-17. https://doi.org/10.1128/AAC.01892-17.

Address correspondence to Bruno Pradines, bruno.pradines@gmail.com.

TABLE 1 Genotyping of two samples obtained from a patient from 2014 and 2016 using eight polymorphic microsatellite markers specific for *P. falciparum*

Sample yr	Microsatellite allele length[a] (bp) for:							
	PfPK2	TA109	Poly α	ARA2	TA42	Pfg377	TA1	TA81
2014	162.21	**166.56**	156.44	67.72	**191.43**	101.68	169.26	121.73
	168.21	169.58	**159.65**	**73.60**	206.12	**107.40**		**136.52**
	177.03	181.51	168.83					
	191.60		174.94					
2016	**177.03**	**166.56**	**159.65**	**73.59**	**191.38**	**107.41**	166.26	**136.63**

[a]Alleles present in both samples are shown in boldface.

asthenia. Since her last malaria manifestation in 2014, the patient did not travel to an area where malaria was endemic and did not receive any blood transfusions. Nevertheless, she tested positive for malaria by microscopy, with 0.75% parasitemia by *P. falciparum*, including 50% late trophozoites. The unexpected diagnosis was confirmed in Bordeaux by real-time PCR using the FTD malaria diagnostic test and by the Centre National de Référence du Paludisme in Marseille by real-time PCR using species-specific primers and probes against the aquaglyceroporin gene of *P. falciparum*. No other infection was documented. She did not support a first intake of atovaquone-proguanil (Malarone) and was perfused with a single dose of intravenous artesunate, followed by a consecutive 3-day dihydroartemisinin-piperaquine oral treatment. The patient recovered well and delivered her baby under normal conditions.

To confirm the recrudescence of *P. falciparum* infection, the genetic diversity of blood samples from 2014 and 2016 was assessed using multilocus variable-number tandem-repeat analysis. These samples were genotyped using eight polymorphic microsatellite markers specific for *P. falciparum* (poly α, TA109, TA1, TA81, TA42, ARA2, PfPK2, and Pfg377). Microsatellite amplification was performed as previously described (3). To determine lengths, PCR products were analyzed using an ABI 3130XL capillary sequencer (Applied Biosystems, Foster City, CA). Microsatellite allele length was determined using internal size standards (GeneScan 500 LIZ size standard, Applied Biosystems) via GeneMapper v4.0 software (Applied Biosystems). Diversity of the *P. falciparum* samples was analyzed by comparing the length variation of polymorphic microsatellite markers between the 2014 and 2016 samples (Table 1). The two samples were successfully genotyped using the eight specific polymorphic microsatellite markers. The number of alleles per microsatellite marker varied from 1 to 4 for the 2014 sample; only 1 allele per microsatellite marker (i.e., a unique genotypic profile) was found in the 2016 sample. The initial *P. falciparum* population contained at least four parasitic clones, whereas a single clone was found in the 2016 sample. This clone contained 7 identical alleles from the initial *P. falciparum* infection of 2014. The allele length was different for the TA1 locus; a 3-bp difference for locus TA1 can define a distinct clone. Only one clone was identified for locus TA1 in the initial isolate. However, allele differences between the initial and recrudescent parasites were already shown, probably due to undetectable parasites present in the initial isolate (4). The eight microsatellite markers were genotyped three times for each sample to ensure reproducibility of the results. Very low levels of polymorphisms were found between the *P. falciparum* populations obtained from the patient in 2014 and 2016. The isolate clonality for the eight microsatellites, the identity of the fragment sizes for seven microsatellites, and the absence of travel to an area where malaria is endemic suggested that the clone from 2016 was recrudescent from the strain of 2014 and did not result from a new infection by a mosquito bite.

The *ex vivo* susceptibility to antimalarial drugs was evaluated for the two samples as previously described (5). Additionally, the K13 propeller gene involved in artemisinin resistance, *pfmdr1* (*P. falciparum* multidrug resistance 1) gene, and *pfcrt* (*P. falciparum* chloroquine resistance transporter) gene involved in quinoline resistance were se-

TABLE 2 *In vitro* susceptibility to standard antimalarial drugs of the initial *P. falciparum* isolate from 2014 versus *P. falciparum* W2 clone tested with the same plate batch

Drug	IC_{50}[a]		Resistance cutoff
	2014 Isolate	W2 clone (SD)	
Piperaquine (nM)	174	53.8 (9.0)	135
Dihydroartemisinin (nM)	6.3	2.6 (0.6)	10.5
Artesunate (nM)	2.7	1.5 (0.3)	10.5
Chloroquine (nM)	126	483 (30)	100
Quinine (nM)	434	388 (30)	800
Monodesethylamodiaquine (nM)	38.4	92 (16)	80
Lumefantrine (nM)	18.6	0.93 (0.39)	150
Pyronaridine (nM)	24.8	21.0 (2.8)	60
Mefloquine (nM)	90.9	25.5 (3.2)	30
Doxycycline (μM)	37.0	11.3 (2.1)	35

[a]IC_{50}s of W2 are means of 7 independent experiments. Threshold values for reduced *in vitro* susceptibility or resistance were estimated previously (5, 20).

quenced as previously described (6). The copy number amplification of *pfmdr1*, involved in mefloquine resistance, was assessed as previously described (7). To explore piperaquine resistance, plasmepsin II copy number amplification and single nucleotide polymorphisms in *pfcrt* (codons 145 and 350) and in the exonuclease gene (PF3D7_1362500, codon 415) were assessed (8–11). The sample from 2014 was found to be highly resistant to piperaquine 174 nM and mefloquine 90.9 nM and weakly resistant to chloroquine 126 nM and doxycycline 37 μM (Table 2) *in vitro*. The sample from 2016 failed to cultivate. Sequencing of the two samples revealed wild-type 72-76 haplotype (CVMNK) and no mutations at codons 145 (F145) and 350 (C350) for the *pfcrt* gene, wild-type haplotype for the *pfmdr1* gene (N86, Y184, S1034, N1046, and D1246 alleles), wild-type exonuclease gene, and wild-type K13. The wild-type 72-76 haplotype may explain the weak *in vitro* chloroquine resistance. The two samples also showed only one copy for *pfmdr1* and plasmepsin II genes.

In most *P. falciparum* infections, the incubation period between an infected mosquito bite and symptomatic malaria is ~9 to 30 days. Clinical manifestations of *P. falciparum* malaria 2 to 9 years after infection were previously described (2, 12, 13). Generally, recurrence of *P. falciparum* infection in Europe affects (i) migrants from areas where malaria is endemic in whom malaria parasites were not initially detected (1); (ii) patients with immunocompromised conditions, such as pregnancy or coinfection with HIV or sickle cell disease (13, 14); and (iii) patients undergoing chemoprophylaxis (5, 15).

In our clinical case, malaria symptoms with fever developed in a pregnant patient 2 years after exposure to malaria during a trip to Cameroon in 2014 and treatment of a *P. falciparum* infection with dihydroartemisinin-piperaquine. This patient was infected in 2016 with the same strain as that found in 2014 and, more specifically, with one *P. falciparum* clone identified in the initial polyclonic sample of 2014. In contrast to *Plasmodium vivax* and *Plasmodium ovale* infections, *P. falciparum* has no hypnozoite form that causes relapse after several years. A specific localization of *P. falciparum* parasites causing relapse is not known. One of the most likely hypotheses explaining recrudescence in this patient is that the dihydroartemisinin-piperaquine treatment in 2014 did not completely clear the infection, and one clone survived from the initial parasite population. The polyclonal parasites from 2014 showed reduced susceptibility to piperaquine but not to artemisinin derivatives. Additionally, no K13 mutation involved in artemisinin resistance and no polymorphism (mutation or copy number amplification) in Asia was observed in either of the samples. However, these data on artemisinin resistance should be considered with caution; the standard *in vitro* test we used is not well adapted to monitor resistance to artemisinin derivatives, and K13 mutation is not yet associated with artemisinin resistance in Africa, in contrast to that in Asia. Additionally, the molecular markers involved in piperaquine resistance in Asia are not yet validated in Africa. Recent publications reported that parasites collected from African patients still parasitemic at day 3 and beyond after artemether-lumefantrine

treatment did not carry mutation on K13 (16). In our case, the patient originated from Cameroon and reported many malarial infections during her childhood, thus leading to the development of partial immunity against malaria parasitemia and symptoms. Indeed, indigenous people acquire a naturally partial and unstable protective immunity against symptoms related to *P. falciparum*, called premunity, which is mainly associated with repeated infections (17). In the case of this Cameroonian patient, premunity acquired during childhood probably prevented the onset of severe clinical symptoms for 2 years. In 2016, the pregnancy of the patient may have caused decreased immunity, resulting in elevated parasitemia- and malaria-associated symptoms. The pregnancy state creates an antigenic variability in the placenta (18), and the switching of parasite surface antigens allows malaria parasites to escape the immune response, resulting in an increase in parasitemia. This clinical case of *P. falciparum* malaria, manifested 2 years after exposure and after the first-treated uncomplicated infection, raises questions about the chronicity and activation of *P. falciparum* infection. The presence of late trophozoites (50%), which are not normally detected in the peripheral blood of patients, suggests altered sequestration. Recent studies suggested that *P. falciparum* infection may induce dormant stages, such as persisting nidus of sequestered parasites described in the placenta (19). Some recurrences of *P. falciparum* malaria may be caused by dormant erythrocytic forms that are newly activated as a consequence of decreased immunity.

Therefore, *P. falciparum* infection should not be excluded in patients with symptoms of malaria, even a long time after travel to an area where malaria is endemic, especially when patients are under mild immunosuppression, as occurs with pregnancy.

Ethics statement. Bio-banking of human clinical samples used for malaria diagnostics and secondary uses for scientific purposes is possible as long as the corresponding patients are informed and have not indicated any objections. This requirement was fulfilled here by giving verbal information to the patient, and no immediate or delayed patient opposition was reported to the hospital clinicians. Informed consent was not required for this study because the sampling procedures and testing are part of the French national recommendations for the care and surveillance of malaria.

ACKNOWLEDGMENTS

We thank the patients, the staff of the Hospital Center of Bordeaux and Rémy Amalvict, and Nicolas Benoit and Joel Mosnier from the Unit of Parasitology and Entomology for technical support.

This study was funded by the French Institute for Public Health Surveillance (Santé Publique France, grant CNR paludisme).

We declare that we have no competing interests.

REFERENCES

1. D'Ortenzio E, Godineau N, Fontanet A, Houze S, Bouchaud O, Matheron S, Le Bras J. 2008. Prolonged *Plasmodium falciparum* infection in immigrants, Paris. Emerg Infect Dis 14:323–326. https://doi.org/10.3201/eid1402.061475.
2. Theunissen C, Janssens P, Demulder A, Nouboussié D, Van-Esbroeck M, Van-Gompel A, Van-Denende J. 2009. Falciparum malaria in patient 9 years after leaving malaria-endemic area. Emerg Infect Dis 15:115–116. https://doi.org/10.3201/eid1501.080909.
3. Anderson TJC, Haubold B, Williams JT, Estrada-Franco JG, Richardson L, Mollinedo R, Bockarie M, Mokili J, Mharakurwa S, French N, Whitworth J, Velez ID, Brockman AH, Nosten F, Ferreira MU, Day KP. 2000. Microsatellite markers reveal a spectrum of population structures in the malaria parasite *Plasmodium falciparum*. Mol Biol Evol 17:1467–1482. https://doi.org/10.1093/oxfordjournals.molbev.a026247.
4. Savini H, Bogreau H, Bertaux L, Bouchiba H, Kraemer P, Parzy D, Garnotel E, Rogier C, Simon F, Pradines B. 2008. First case of emergence of atovaquone-proguanil resistance in *Plasmodium falciparum* during treatment in a traveler in Comoros. Antimicrob Agents Chemother 52:2283–2284. https://doi.org/10.1128/AAC.00282-08.
5. Javelle E, Madamet M, Gaillard T, Velut G, Surcouf C, Michel R, Garnotel E, Simon F, Pradines B. 2016. Delayed *P. falciparum* malaria after doxycycline prophylaxis, Central African Republic. Antimicrob Agents Chemother 60:2592–2593. https://doi.org/10.1128/AAC.01858-15.
6. Roseau JB, Pradines B, Paleiron N, Vedy S, Madamet M, Simon F, Javelle E. 2016. Failure of dihydroartemisinin plus piperaquine treatment of falciparum malaria by under-dosing in an overweight patient. Malar J 15:479. https://doi.org/10.1186/s12936-016-1535-8.
7. Wurtz N, Fall B, Pascual A, Diawara S, Sow K, Baret E, Diatta B, Fall KB, Mbaye PS, Fall F, Diémé Y, Rogier C, Bercion R, Briolant S, Wade B, Pradines B. 2012. Prevalence of molecular markers of *Plasmodium falciparum* drug resistance in Dakar, Senegal. Malar J 11:197. https://doi.org/10.1186/1475-2875-11-197.
8. Witkowski B, Duru V, Khim N, Ross LS, Saintpierre B, Beghain J, Chy S, Kim S, Ke S, Kloeung N, Eam R, Khean C, Ken M, Loch K, Bouillon A, Domergue A, Ma L, Bouchier C, Leang R, Huy R, Nuel G, Barale JC, Legrand E, Ringwald P, Fidock DA, Mercereau-Puijalon O, Ariey F, Ménard D. 2017. A surrogate marker of piperaquine-resistant *Plasmodium falciparum* malaria: a phenotype-genotype association study. Lancet Infect Dis 17:174–183. https://doi.org/10.1016/S1473-3099(16)30415-7.
9. Agrawal S, Moser KA, Morton L, Cummings MP, Parihar A, Dwivedi A,

Shetty AC, Drabek EF, Jacob CG, Henrich PP, Parobek CM, Jongsakul K, Huy R, Spring MD, Lanteri CA, Chaorattannakawee S, Lon C, Fukuda MM, Saunders DL, Fodock DA, Lin JT, Juliano JJ, Plowe CV, Silva JC, Takala-Harrison S. 2017. Association of a novel mutation in the *Plasmodium falciparum* chloroquine resistance transporter with decreased piperaquine sensitivity. J Infect Dis 216:468–476. https://doi.org/10.1093/infdis/jix334.

10. Pelleau S, Moss EL, Dhingra SK, Volney B, Casteras J, Gabryszwski SJ, Volkman SK, Wirth DF, Legrand E, Fidock DA, Neafsey DE, Musset L. 2015. Adaptative evolution of malaria parasites in French Guiana: reversal of chloroquine resistance by acquisition of a mutation in *pfcrt*. Proc Natl Acad Sci U S A 112:11672–11677. https://doi.org/10.1073/pnas.1507142112.
11. Amato R, Lim P, Miotto O, Amaratunga C, Dek D, Pearson RD, Almagro-Garcia J, Neal AT, Sreng S, Suon S, Drury E, Jyothi D, Stalker J, Kwiatkowski DP, Fairhurst RM. 2017. Genetic markers associated with dihydroartemisinin-piperaquine failure in *Plasmodium falciparum* malaria in Cambodia: a genotype-phenotype association study. Lancet Infect Dis 17:164–173. https://doi.org/10.1016/S1473-3099(16)30409-1.
12. Berrevoets MA, Sprong T, Meis JF, Dofferhoff AS. 2013. *Plasmodium falciparum* malaria recrudescence occurring 2.5 years after leaving an endemic country. Neth J Med 71:426–428.
13. Greenwood T, Vikerfors T, Sjöberg M, Skeppner G, Färnert A. 2008. Febrile *Plasmodium falciparum* malaria 4 years after exposure in a man with sickle cell disease. Clin Infect Dis 47:e39–e41. https://doi.org/10.1086/590250.
14. Giobbia M, Tonon E, Zanatta A, Cesaris L, Vaglia A, Bisoffi Z. 2005. Late recrudescence of *Plasmodium falciparum* malaria in a pregnant woman: a case report. Int J Infect Dis 9:234–235. https://doi.org/10.1016/j.ijid.2004.08.002.
15. Day JH, Behrens RH. 1995. Delay in onset of malaria with mefloquine prophylaxis. Lancet 345:398.
16. Madamet M, Kounta MB, Wade KA, Lo G, Diawara S, Fall M, Bercion R, Nakoulima A, Fall KB, Benoit N, Gueye MW, Fall B, Diatta B, Pradines B. 2017. Absence of association between polymorphisms in the K13 gene and the presence of *Plasmodium falciparum* parasites at day 3 after treatment with artemisinin derivatives in Senegal. Int J Antimicrob Agents 49:754–756. https://doi.org/10.1016/j.ijantimicag.2017.01.032.
17. Hviid L. 2005. Naturally acquired immunity to *Plasmodium falciparum* malaria in Africa. Acta Trop 95:270–275. https://doi.org/10.1016/j.actatropica.2005.06.012.
18. Bancells C, Deitsch KW. 2013. A molecular switch in the efficiency of translation reinitiation controls expression of *var2csa*, a gene implicated in pregnancy-associated malaria. Mol Microbiol 90:472–488. https://doi.org/10.1111/mmi.12379.
19. Muehlenbachs A, Mutabingwa TK, Fried M, Duffy PE. 2007. An unusual presentation of placental malaria: a single persisting nidus of sequestered parasites. Hum Pathol 38:520–523. https://doi.org/10.1016/j.humpath.2006.09.016.
20. Pascual A, Madamet M, Briolant S, Gaillard T, Amalvict R, Benoit N, Travers D, Pradines B. 2015. Multinormal *in vitro* distribution of *Plasmodium falciparum* susceptibility to piperaquine and pyronaridine. Malar J 14:49. https://doi.org/10.1186/s12936-015-0586-6.