



Etudes des mécanismes cellulaires et moléculaires de *Plasmodium falciparum* impliqués dans les résistances aux combinaisons à bases de dérivés de l'artémisinine

Valentine Duru

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Valentine Duru. Etudes des mécanismes cellulaires et moléculaires de *Plasmodium falciparum* impliqués dans les résistances aux combinaisons à bases de dérivés de l'artémisinine. Microbiologie et Parasitologie. Université Montpellier, 2016. Français. NNT : 2016MONTT104 . tel-01615022

HAL Id: tel-01615022

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THÈSE

Pour obtenir le grade de
Docteur

Délivré par l'Université de Montpellier

**Préparée au sein de l'école doctorale GAIA
Et de l'unité de recherche UPR - IPC - Institut Pasteur
Cambodge**

Spécialité : BDI - Biologie des Interactions

Présentée par Valentine DURU

**Études des mécanismes cellulaires et
moléculaires de *Plasmodium falciparum*
impliqués dans les résistances aux combinaisons
à base de dérivés de l'artémisinine**

Soutenue le 15 décembre 2016 devant le jury composé de

M. Didier MENARD, DR, Institut Pasteur	Directeur de thèse
M. Rémy DURAND, MCU-PH, Université Paris 13	Rapporteur
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UNIVERSITÉ DE MONTPELLIER

UPR - IPC - Institut Pasteur Cambodge

Année 2016

N° attribué par la bibliothèque

.....

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Ecole Doctorale : GAIA - Biodiversité, Agriculture, Alimentation, Environnement, Terre, Eau

Spécialité : BDI - Biologie des Interactions

Présentée et soutenue publiquement

par

Valentine DURU

Le 15 décembre 2016

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Directeur de thèse

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*À mes grands-pères, Antoine et André
Je suis fière d'être votre petite-fille
Vous me manquez énormément.*

Remerciements

Je tiens tout d'abord à remercier vivement mon Directeur de Thèse, le Docteur Didier Ménard, pour m'avoir accueillie dans son unité et m'avoir offert l'opportunité de réaliser un projet de thèse aussi riche qu'intéressant. Merci de vos conseils, de votre soutien, et de votre confiance.

Je remercie ensuite le Docteur Vincent Deubel, ancien directeur de l'Institut Pasteur du Cambodge, et le Docteur Didier Fontenille, son successeur, pour m'avoir ouvert les portes de votre Institut.

Je souhaite également remercier les Docteurs Rémy Durand et Jérôme Clain d'avoir accepté d'évaluer mes travaux de thèse en qualité de rapporteurs et de siéger dans mon jury de soutenance.

Un grand merci aux Professeurs Antoine Berry et Stéphane Picot, et aux Docteurs Anna Cohuet, Léonardo Basco et Jean-Christophe Barale d'avoir accepté de faire partie de mon jury de thèse.

Je tiens également à remercier les Docteurs Odile Mercereau-Puijalon et Frédéric Ariey pour leur implication tout au long de ma thèse et les collaborations qui en ont découlés, et pour avoir accepté de venir siéger à ma soutenance en tant qu'invités.

Merci également à Benoît, d'avoir eu la patience de me former aux techniques de biologie cellulaire, et d'avoir toujours été là par la suite, pour les discussions et les manips de nuit, pour les victoires comme pour les coups durs, et pour le reste.

Je remercie également du fond du cœur mes collègues de bureau : merci Nimol de m'avoir aidé à m'intégrer, à grand renfort de cours de khmer et de *num akaor* ; merci Laura d'avoir eu

l'esprit assez aventureux pour venir me rejoindre ; merci Amélie pour nos remakes de « My Drunk Kitchen » ; et merci Jean, sur qui on peut toujours compter pour les bières du vendredi soir, pour ne citer qu'elles (mais pas pour la cafetière !). Merci à vous pour tous ces moments de décompression.

Thank you Nimol and Vatey, for your skills, your patience, your help, and your kindness ; it's been a real pleasure to work by your side. *Orkun chran...* And thanks to the whole Malaria Unit team, for their welcome, their smiles, and their expertise.

J'aimerais également remercier mes professeurs de l'ESBS, en particulier Yves Nominé et Vincent Phalip, pour leurs enseignements et leur bienveillance. Un grand merci également à Benoît Quilichini et à toute l'équipe de cytogénétique acquise de BIOMNIS, avec qui j'ai pu faire mes premiers pas dans l'univers des laboratoires et de la santé. C'est grâce à vous tous que j'ai eu la volonté et le courage de me lancer dans l'aventure du doctorat.

Enfin, un immense merci à ma famille. À mes grands-mères, pour votre sagesse et votre force. À ma mère et Aurélien, à mon père et Karine, merci pour votre amour, votre soutien inconditionnel, votre présence même à 10 000 km de distance. Merci à mes sœurs, Fanny, Lison et Lila, pour avoir toujours su me faire rire, même dans les moments difficiles. J'ai de la chance de vous avoir. Un grand merci également à Christine et Rémi pour leur aide précieuse avec mes démarches d'inscription. Vous m'avez tous beaucoup manqué, et j'ai hâte de pouvoir trinquer avec vous.

Et merci à toutes ces belles personnes qui ont croisé ma route durant cette aventure : Anne-Claire, Sayonne, Lydie, Julien, Pauline, Judith, Anthony, Camille, Chanaki, Brandy, Giulia, Savon, Lili et Khmaw...

Résumé en français

Titre: Études des mécanismes cellulaires et moléculaires de *Plasmodium falciparum* impliqués dans les résistances aux combinaisons à base de dérivés de l'artémisinine.

Les combinaisons thérapeutiques à base d'artémisinine (ou CTAs) sont une des clés de voûte des stratégies actuelles de lutte contre le paludisme : ces thérapies ont en effet joué un rôle important dans la réduction de l'impact du paludisme au cours de la dernière décennie. Cependant, ces progrès sont aujourd'hui compromis par l'émergence de parasites *Plasmodium falciparum* résistants aux dérivés de l'artémisinine (ART). Les premiers parasites résistants ont été détectés pour la première fois en 2008 dans l'ouest du Cambodge, puis dans plusieurs pays avoisinants d'Asie du Sud-Est. Le problème de la multirésistance aux antipaludiques s'est récemment aggravé au Cambodge : plusieurs études ont rapporté l'émergence de parasites résistants à la pipéraquine (PPQ), la dernière génération de molécule partenaire utilisée en combinaison avec la dihydroartémisinine, entraînant des taux alarmants d'échecs cliniques. Pour préserver l'efficacité de ces combinaisons, la surveillance et la compréhension de ces deux types de résistance sont cruciales afin d'éviter la dissémination de parasites multirésistants en dehors d'Asie du Sud-Est, et particulièrement en Afrique où les conséquences sanitaires seraient désastreuses. À cette fin, le développement de nouveaux outils est nécessaire pour étudier les mécanismes biologiques et moléculaires impliqués dans la résistance aux CTAs et pour surveiller la distribution géographique des parasites multirésistants.

Dans la première partie de cette thèse, axée sur la résistance à l'artémisinine, nous présentons tout d'abord la découverte de mutations au sein du gène *K13*, marqueur moléculaire pour la résistance à l'ART. Puis nous confirmons leur rôle comme déterminant majeur de cette résistance. Enfin, nous analysons l'étendue de cette résistance au travers d'une cartographie mondiale des polymorphismes de *K13*. Dans la seconde partie de cette thèse, nous présentons nos travaux portant sur l'émergence de la résistance à la pipéraquine au Cambodge, en confirmant dans un premier temps que des parasites multirésistants à l'ART et à la PPQ circulent désormais dans le pays. Puis en détaillant la mise en point d'un nouveau test phénotypique pour la détection des parasites PPQ-résistants, le Piperaquine Survival Assay ou PSA. Pour finir, nous exposons la découverte de l'amplification du gène *PfPM2* comme marqueur moléculaire pour la résistance à la PPQ.

Mots clés: Paludisme; *Plasmodium falciparum*; Résistance; Cambodge; Artémisinine; Pipéraquine.

Summary in English

Title: Study of cellular and molecular mechanisms of *Plasmodium falciparum* involved in Artemisinin-based Combination Treatment resistance.

Artemisinin-based combination therapies (ACTs) are one of the pillars of the current strategies implemented for fighting malaria. Over the last decade, ACTs have played a major role in decreasing malaria burden. However, this progress is being jeopardized by the emergence of artemisinin-resistant *Plasmodium falciparum* parasites. Artemisinin (ART) resistance was first detected in western Cambodia in 2008 and has since been observed in neighboring countries in Southeast Asia. The problem of antimalarial drug resistance has recently worsened in Cambodia, with reports of parasites resistant to piperaquine (PPQ), the latest generation of partner drug used in combination with dihydroartemisinin, leading to worrying rates of clinical treatment failure. The monitoring and the comprehension of both types of resistance are crucial to prevent the spread of multi-drug resistant parasites outside Southeast Asia, and particularly to Africa, where the public health consequences would be catastrophic. To this end, new tools are required for studies of the biological and molecular mechanisms underlying resistance to antimalarial drugs and for monitoring the geographic distribution of the resistant parasites.

In the first section of this thesis, centered on artemisinin resistance, we first present the discovery of mutations within the *K13* gene as a molecular marker for ART resistance. Then we confirm their role as a major determinant of such a resistance. Finally, we analyze the extent of ART resistance through a global mapping of *K13* polymorphisms. In the second section, we present our work on the emergence of piperaquine resistance in Cambodia, by initially confirming that multiresistant parasites now circulate in the country. Then we detail the development of a new phenotypical test for the detection of PPQ-resistant parasites, the Piperaquine Survival Assay or PSA. Lastly, we report the discovery of the *PfPM2* gene amplification as a candidate molecular marker for PPQ-resistance.

Key words: Malaria; *Plasmodium falciparum*; Resistance; Cambodia; Artemisinin; Piperaquine.

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Synthèse bibliographique



1. Epidémiologie du paludisme

Le paludisme est une maladie infectieuse potentiellement mortelle, causée par des parasites du genre *Plasmodium*, transmis à l'Homme par la piqûre de moustiques femelles infestées du genre *Anopheles*. Bien qu'il existe plus de 400 espèces d'anophèle, seules une soixantaine sont vectrices du paludisme^{1,2}.

La transmission du paludisme est active dans 95 pays, contre 106 en 2000. D'après l'OMS, 21 pays seraient en mesure d'éliminer le paludisme d'ici 2020³ (Fig. 1).

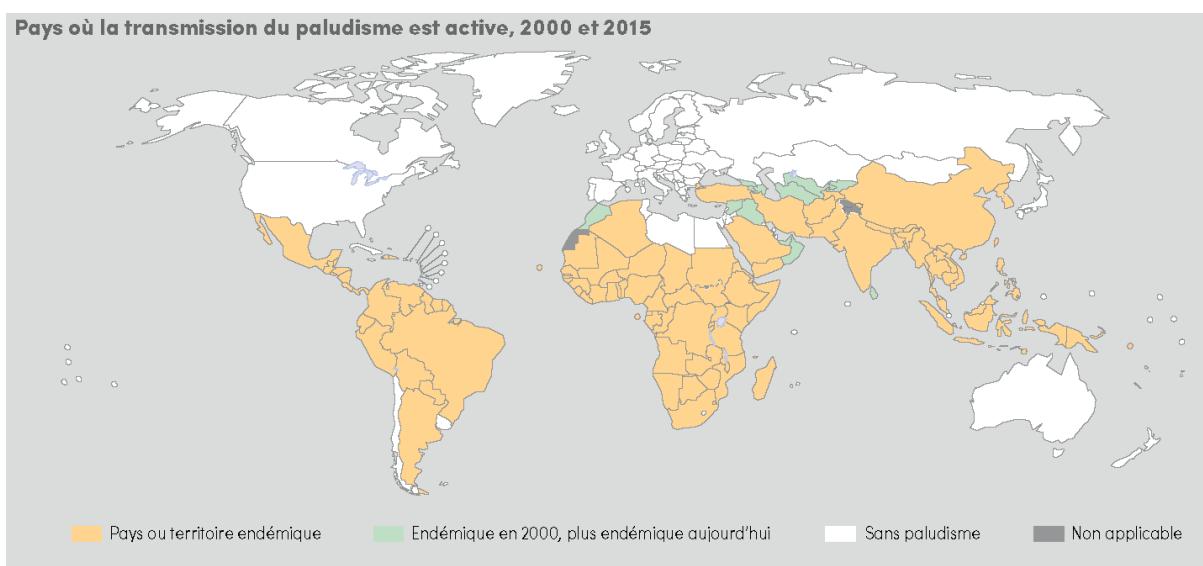


Figure 1. Pays où la transmission du paludisme est active, 2000 et 2015 (source : OMS)

Le paludisme est un problème majeur de santé publique, avec 3,2 milliards de personnes considérées à risque, soit la moitié de la population mondiale. On estime à 214 millions (plage comprise entre 149 et 303 millions) le nombre de cas en 2015, dont 88% en Afrique sub-saharienne et à 438 000 (plage comprise entre 236 000 et 635 000) le nombre de décès, dont 90% en Afrique sub-saharienne³.

Certains groupes de population sont plus vulnérables à une telle infection : les femmes enceintes, les nourrissons, les enfants de moins de 5 ans, les personnes porteuses du VIH ou

atteintes du sida, les migrants non immunisés, les populations itinérantes et les voyageurs. Ces personnes sont plus à risque de développer une forme sévère de la maladie ; à titre d'exemple, 70% des décès surviennent chez les enfants de moins de 5 ans³.

Les régions où les infections ont lieu tout au long de l'année sont qualifiées de zones à paludisme stable. En Afrique subsaharienne, la transmission est intense mais largement confinée aux 3-4 mois de saison des pluies. Dans de telles conditions, la morbidité et la mortalité sont prononcées lors de la petite enfance, mais l'immunité acquise à l'âge adulte implique que la majorité des infections paludiques restent asymptomatiques^{4, 5}. Dans les régions de faible transmission (qualifiées de zones à paludisme instable), une immunité complète n'est jamais acquise et les infections symptomatiques peuvent survenir à tout âge.

Bien qu'il existe 5 espèces de *Plasmodium* capables d'infecter l'Homme (*P. falciparum*, *P. vivax*, *P. ovale*, *P. malariae* et *P. knowlesi*), deux d'entre elles sont majoritaires :

- ➔ *P. falciparum*, le plus rependu sur le continent Africain⁶ et responsable de la grande majorité des cas mortels.
- ➔ *P. vivax*, espèce dominante dans les zones d'endémie en dehors de l'Afrique sub-saharienne⁷, rarement létale mais provoquant des rechutes périodiques ('relapse').

2. Biologie du parasite

Les *Plasmodium* sont des protozoaires de l'embranchement des *Apicomplexa*.

Leur cycle de développement est dit « dixène », c'est-à-dire nécessitant deux hôtes : la phase asexuée se déroule chez l'Homme, hôte intermédiaire ou réservoir, alors que la phase sexuée a lieu chez la femelle moustique du genre *Anopheles*, hôte définitif (Fig. 2).

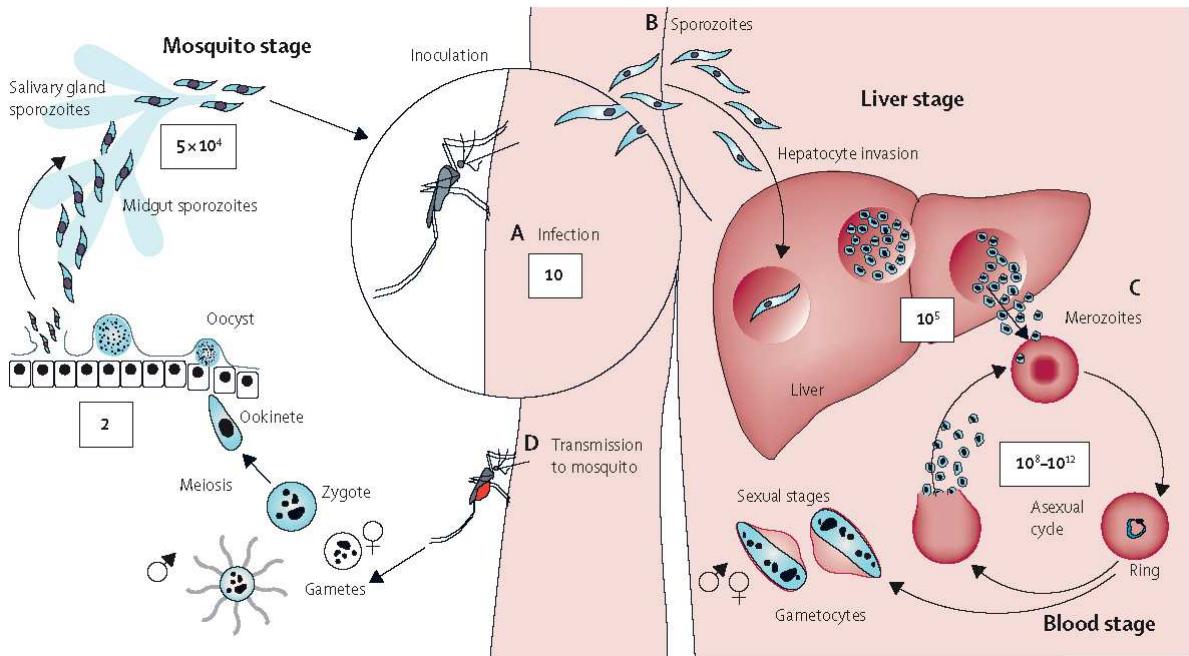


Figure 2. Cycle de développement de *Plasmodium falciparum*⁸

Lors d'un repas sanguin, le moustique infecté injecte des sporozoites qui atteignent et envahissent rapidement les hépatocytes. Une fois à l'intérieur de ces derniers, le parasite se développe durant 5 à 8 jours jusqu'au stade de schizonte, qui en se rompant libère des merozoites dans la circulation sanguine. Cette phase primaire, dite phase hépatique, est asymptomatique. Chez *P. vivax*, certains parasites peuvent rester dans le foie sous forme dormante durant des semaines, voire des mois ou des années⁹: ces formes sont appelées «hypnozoites» et sont responsables des rechutes fréquemment observées après une infection à *P. vivax*.

Les merozoites ainsi libérés (10 000 à 30 000 par schizont hépatique chez *P. falciparum*) envahissent les globules rouges, marquant l'entrée en phase secondaire, dite érythrocytaire, qui est la phase symptomatique de l'infection. Les parasites se développent dans les érythrocytes en 48h, progressant par les stades ring (0-24h), trophozoite (24-36h) et schizonte (36-48h), jusqu'à libérer de nouveaux merozoites. Ceci permet une progression rapide de

l'infection puisque chez *P. falciparum*, un schizonte produit entre 16 et 32 merozoites, capables d'envahir à leur tour de nouveaux érythrocytes (Fig.3). Chez un individu non-immun, la population parasitaire augmente entre 6 et 20 fois par cycle¹⁰.

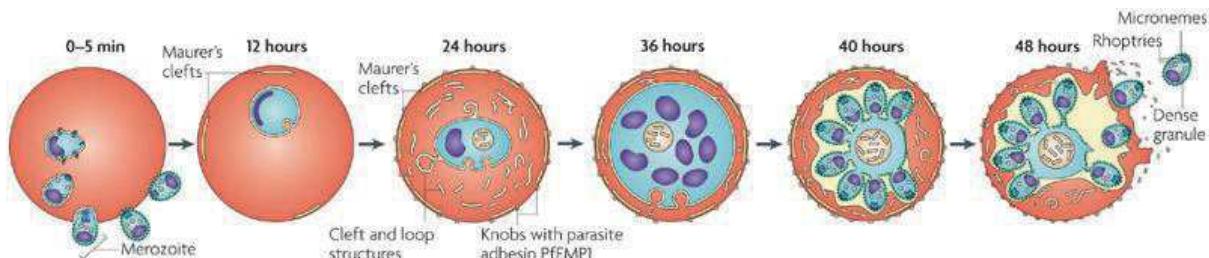


Figure 3. Stades érythrocytaires de *Plasmodium falciparum*¹¹

Au cours de ces cycles successifs d'invasion de nouveaux globules rouges et de libération de merozoites, certains parasites se différencient en gamétocytes mâles ou femelles (en 9 à 11 jours pour *P. falciparum*). La gamétoctogenèse passe par 5 stades successifs de développement, dont seul le dernier (stade V) est responsable de la transmission de la maladie. En effet, en se nourrissant sur un être humain infecté, la femelle anophèle ingère du sang contenant des gamétocytes mâles et femelles au stade V et s'infecte à son tour. La fécondation a alors lieu dans le tractus digestif, conduisant à la production massive de sporozoites (jusqu'à 20 000 sporozoites par sporogonie). Ce sont ces sporozoites qui, en rejoignant les glandes salivaires, pourront être transmis à un nouvel être humain lors d'un futur repas sanguin.

3. Signes cliniques de la maladie

Le paludisme est ce que l'on appelle une affection fébrile aigue. Les symptômes apparaissent généralement une dizaine de jours après la piqûre infectieuse chez les personnes non immunisées, mais la période d'incubation peut être beaucoup plus longue chez les sujets ayant acquis un certain degré d'immunité.

Les manifestations cliniques sont très diverses et aspécifiques, correspondant à un symptôme pseudo-grippal, ce qui rend le seul diagnostic clinique peu fiable : fièvre, maux de tête, douleurs musculaires, affaiblissement, vomissements, diarrhées, toux. C'est l'accès palustre « simple ».

Ces accès palustres sont caractérisés par des cycles de 2-3 jours alternant frissons, sueurs et fièvre, synchronisés avec le cycle de développement du parasite et l'éclatement des globules rouges.

Si un traitement adapté n'est pas administré à temps, la maladie peut rapidement évoluer en une forme plus sévère potentiellement mortelle, ou accès palustre « pernicieux »: on peut alors observer des signes beaucoup plus inquiétants, tels qu'une anémie due à l'éclatement des érythrocytes infectés¹², une acidose lactique associée à de l'hypoglycémie¹³, une défaillance de certains organes (souvent une détresse respiratoire¹⁴), un neuropaludisme entraîné par la séquestration des érythrocytes infectés dans les capillaires cérébraux¹⁵...

Certains individus, à force d'infections répétées, développent une immunité naturelle dite « immunité acquise ». Ils ne déclarent alors pas forcément de signes cliniques mais deviennent porteurs asymptomatiques, et peuvent potentiellement transmettre la maladie. Ces porteurs asymptomatiques constituent un réservoir qui ne doit pas être négligé dans une optique d'élimination du paludisme.

4. Moyens de lutte pour le contrôle du paludisme

Entre 2000 et 2015, l'incidence du paludisme (nombre de nouveaux cas parmi les populations exposées) a baissé de 37% à l'échelle mondiale tandis que le taux de mortalité a reculé de 60% : environ 6,2 millions de décès auraient été évités depuis 2001³. Ces progrès

s'expliquent par une augmentation substantielle des moyens financiers (financement mondial de la lutte contre le paludisme a augmenté de 960 millions US\$ en 2005 à 2,5 milliards US\$ en 2014), qui ont permis une intensification des efforts de lutte.

Tout d'abord, la lutte antivectorielle demeure le moyen principal pour limiter la transmission du paludisme (Fig. 4A et 4B) : la distribution de moustiquaires imprégnées d'insecticide et la pulvérisation d'insecticides à effet rémanent à l'intérieur des habitations (efficacité de 3 à 6 mois) se sont révélées des solutions à la fois efficaces et rentables pour réduire l'incidence du paludisme dans les régions endémiques^{16, 17}. En effet, ces mesures ont majoritairement (à hauteur de 68% pour les moustiquaires imprégnées) contribué à la réduction des cas de paludisme en Afrique entre 2000 et 2015¹⁸. Toutefois, une résistance aux 4 classes d'insecticides utilisées a été détectée chez toutes les principales espèces vectrices¹⁹ : depuis 2010, une soixantaine de pays ont rapporté une résistance à au moins une classe d'insecticides. Des mesures urgentes de détection et de surveillance doivent être prises afin de maîtriser la résistance des vecteurs aux insecticides et ainsi éviter une potentielle hausse de l'incidence du paludisme et de la mortalité palustre^{20, 21}.

Il est également fréquemment conseillé aux voyageurs de se protéger au moyen d'un traitement prophylactique²². Il existe pour se faire quatre traitements possibles : la chloroquine (Nivaquine® 100), la méfloquine (Lariam® 250), la doxycycline (Doxypalu® ou Doxy®) et l'association atovaquone - proguanil (Malarone®). Le choix du traitement dépend du pays visité, de la durée du séjour et des caractéristiques individuelles de chaque voyageur, mais le principe reste le même: tuer les stades sanguins (et les stades hépatiques dans le cas de l'association atovaquone – proguanil²³) afin d'éviter que la maladie ne se déclare.

Dans certaines zones endémiques où la transmission du paludisme est de modérée à intense, l'OMS recommande les traitements préventifs intermittents (TPI). Les TPI visent à traiter et

prévenir les accès palustres chez certains groupes de population à risque, en particulier les femmes enceintes, mais également les nourrissons ou les jeunes enfants²⁴. L'intervention consiste en un protocole thérapeutique complet d'antipaludiques (sulfadoxine-pyriméthamine, amodiaquine ou plus récemment combinaisons thérapeutiques à base d'artémisinine - CTA) administrés périodiquement (par exemple lors de visites prénatales systématiques pour les femmes enceintes ou une fois par mois durant la saison des pluies chez les écoliers), indépendamment de la présence d'une telle infection chez les sujets.

Enfin, la bonne gestion des cas avérés joue un rôle crucial dans la stratégie de contrôle du paludisme. Une prise en charge efficace des malades est nécessaire, puisqu'un bon diagnostic et l'administration d'un traitement adapté permettent de réduire l'intensité de la maladie et d'éviter l'évolution vers une forme sévère (Fig. 4C et 4D). Le diagnostic peut être confirmé soit par microscopie (présence de plasmodes dans le sang du patient), soit par un test diagnostic rapide (ou TDR) de qualité avérée (recherche de marqueurs antigéniques du paludisme dans le sang du patient). Les TDRs les plus répandus sont basés sur la détection de la protéine-2 riche en histidine (HRP-2), produite par le gène *hrp2* du parasite. Différentes études ont montré que la sensibilité et la spécificité de ces tests étaient comprises entre 90 et 95% et 85 et 92% respectivement, en comparaison avec la microscopie²⁵⁻²⁸. Une fois l'infection confirmée, le patient reçoit rapidement un traitement adapté, consistant généralement en une association thérapeutique à base d'artémisinine. Cependant, l'émergence de parasites présentant une délétion du gène *hrp2*, entraînant de faux résultats négatifs en TDR²⁹, et de parasites résistants aux traitements menacent ce système efficace de gestion des cas.



Figure 4. Moyens de lutte contre le paludisme. A : Moustiquaire imprégnée d'insecticide ; B : Pulvérisation d'insecticides à effet rémanent à l'intérieur des habitations ; C : Test de diagnostic rapide ; D : Traitement antipaludique de type CTA.

5. Traitements antipaludiques et chimio-résistance

Aujourd’hui, et en l’absence de vaccin efficace, la chimiothérapie antipaludique demeure la seule solution pour traiter le paludisme. Depuis le 19^e siècle, de nombreuses classes de médicaments ont été utilisées comme traitement. Dans les années 50, des molécules synthétiques telles que la chloroquine ont été massivement déployées dans les zones endémiques, avec pour buts ultimes le contrôle du paludisme et son éradication. Cette molécule était particulièrement efficace et a permis pour la première fois un contrôle très satisfaisant du paludisme, en association avec des mesures de contrôles des vecteurs. Malheureusement, l’émergence et la propagation de parasites résistants à la chloroquine (ainsi que de vecteurs résistants aux insecticides) compromirent cette initiative ^{30, 31}. Durant cette période (autour de 1980), d’autres molécules, telles que l’association sulfadoxine-pyriméthamine, furent implémentées, avec une fois encore l’adaptation des parasites comme conséquence ^{32, 33}. La situation sanitaire fut alors critique et l’impact du paludisme à la fin des

années 90 était estimé à 3 millions de morts par an³⁴. Cette progression fut stoppée par l'application de mesures efficaces de contrôle des vecteurs (principalement la distribution à grande échelle de moustiquaires imprégnées) et par l'introduction dans les années 2000 des dérivés d'artémisinine, une nouvelle classe de molécules, comme traitement de première intention du paludisme simple à *P. falciparum*¹⁸.

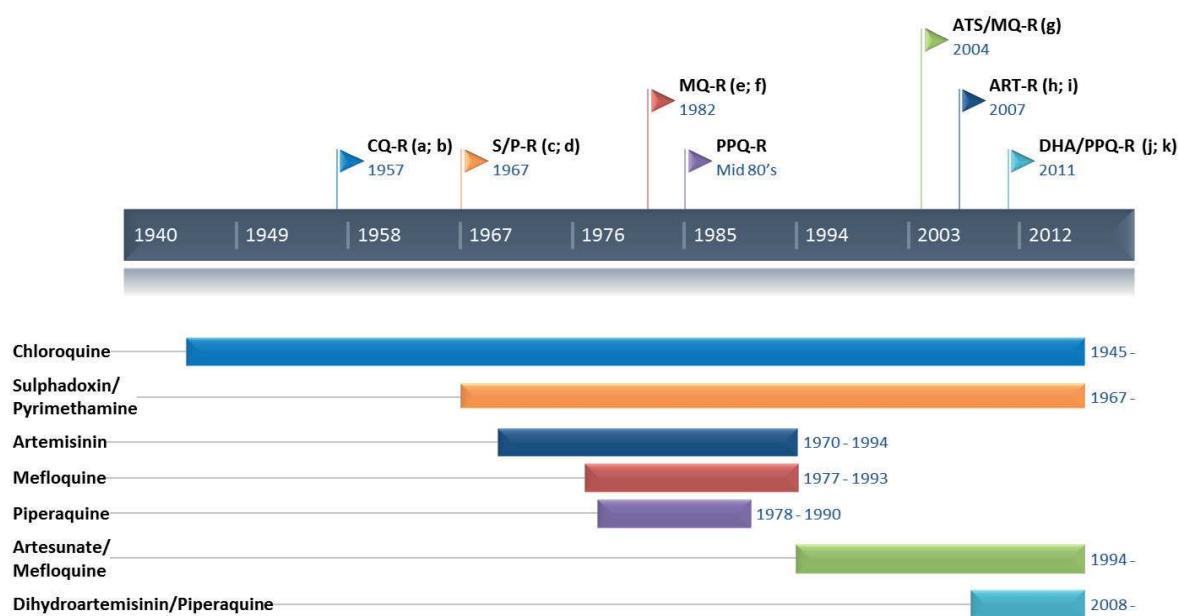


Figure 5. Chronologie de l'introduction des principales molécules antipaludiques et des premiers cas de résistance rapportés pour chacune de ses molécules. Les références citées dans cette figure sont listées ci-dessous :

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6. Artémisinine : découverte et mode d'action

En réponse au besoin urgent de nouvelles molécules antipaludiques, notamment dans le contexte de la guerre du Vietnam, le gouvernement chinois lança en 1967 un programme de recherche secret : le projet 523. Le groupe, mené par le professeur Youyou Tu aujourd’hui lauréate du prix Nobel de Médecine, passa au crible quelques 2000 herbes couramment utilisées dans la pharmacopée traditionnelle chinoise pour le traitement des accès fébriles. L’idée d’utiliser des plantes pour traiter le paludisme n’était alors pas nouvelle, puisque la quinine, extraite du quinquina, était utilisée à cet effet depuis le 17^e siècle. Parmi les herbes étudiées par le groupe de Youyou Tu, un extrait de la plante *Artemisia annua* (Fig. 6A et 6B) montra une grande efficacité contre les stades érythrocytaires de *Plasmodium* : l’artémisinine (ART) ou qinghaosu, le composé responsable de cette activité, fût ainsi découverte et purifiée en 1971 (Fig. 6C)³⁵.

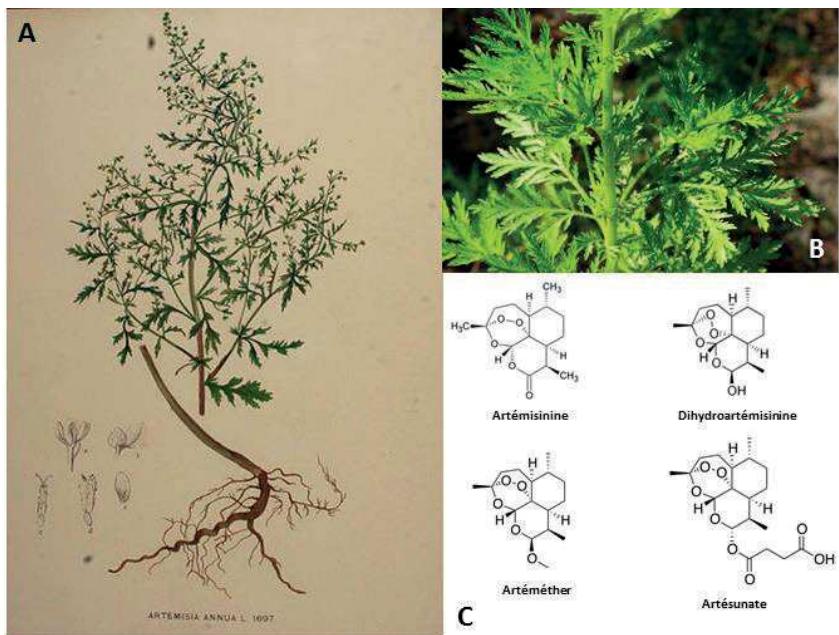


Figure 6. L'artémisinine est extraite de la plante *Artemisia annua*. A : *Artemisia annua*, gravure (source : Kops et al., J., Flora Batava, vol. 22 (1906)); B : *Artemisia annua*, photographie; C : Structures moléculaires des principaux dérivés d'artémisinine

L'artémisinine et l'ensemble de ses dérivés semi-synthétiques sont ce que l'on appelle des prodrogues : ce sont des composés pharmacologiques inactifs qui, une fois administrés, sont métabolisés en un métabolite secondaire actif, la dihydroartémisinine (DHA). L'activité antipaludique de ces lactones sesquiterpéniques semble résider dans le pont endoperoxyde qui les caractérise. Une fois à l'intérieur du parasite, le pont endoperoxyde de la DHA doit être activé par scission « fer-dépendante » afin de générer des radicaux libres³⁶. Ces radicaux libres vont alors réagir avec les groupes vulnérables de certaines protéines ou d'autres biomolécules, entraînant la mort du parasite par stress oxydatif³⁷. Deux voies d'activation du pont endoperoxyde ont été proposées: la première aurait lieu dans les mitochondries³⁸, où l'activation se ferait par la chaîne de transport d'électrons, menant à la production massive de dérivés réactifs de l'oxygène (*Reactive Oxygen Species* ou ROS en anglais); la seconde prendrait place dans la vacuole digestive³⁹ et serait médiée par l'hème (Fe^{2+}) produit par la dégradation de l'hémoglobine, générant des radicaux carbonés actifs.

7. Résistance aux dérivés d'artémisinine

Le phénomène de résistance partielle de *P. falciparum* à l'ART a été rapporté pour la première fois en 2008, dans la province de Battambang à l'ouest du Cambodge. La confirmation arriva en 2009 dans la province de Pailin, épicentre reconnu de la multirésistance de *P. falciparum* aux antipaludiques^{40, 41}. Depuis, la résistance à l'ART a été signalée ailleurs dans la sous-région Bassin du Mékong (à l'ouest du Cambodge, à l'ouest de la Thaïlande, au sud de la Birmanie, au sud du Vietnam)⁴²⁻⁴⁷ et ainsi qu'en Chine⁴⁸ (Fig. 7).



Figure 7. Sites où de l'ART-R a été détectée en 2012 en Asie du Sud-Est (source: WHO 2012)

D'un point de vue clinique, la résistance à l'ART est définie par une clairance parasitaire retardée⁴⁹⁻⁵¹ : une demi-vie de clairance parasitaire ≥ 5 heures a été associée à la résistance à l'ART en Asie du Sud-Est (Fig. 8)⁵². Dans un contexte de transmission intense, tel que rencontré en Afrique subsaharienne, où l'immunité partielle des adultes joue un rôle significatif dans la clairance parasitaire, un seuil adapté doit être défini⁵³.

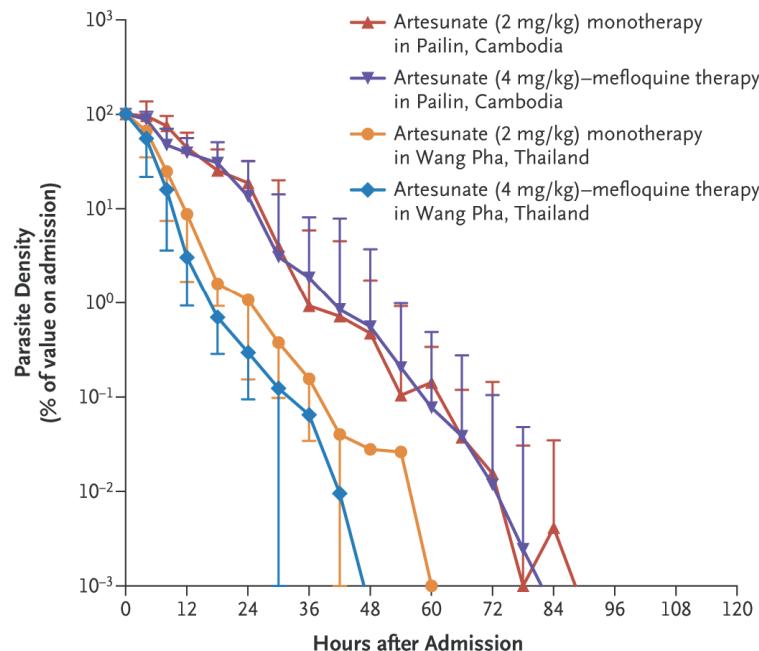


Figure 8. Courbes de clairances parasitaires à Pailin, Cambodge (zone d'ART-R, clairance lente) et Wang Pha, Thaïlande (zone d'ART-S, clairance rapide) après administration d'artesunate ou d'artesunate + MQ⁴³

Actuellement, cette approche est considérée comme la méthode de référence (*gold standard*) mais souffre de plusieurs inconvénients : tout d'abord, les études de clairance parasitaire sont à la fois logistiquement et financièrement astreignantes, puisqu'elles nécessitent le screening de milliers d'individus fébriles afin d'identifier un nombre restreint de patients qui seront hospitalisés plusieurs jours ; ensuite, la clairance parasitaire dépend de nombreux facteurs autres que la résistance intrinsèque des parasites, tels que l'administration du traitement, l'immunité ou encore l'effet pharmacodynamique.

Des approches *in vivo* alternatives ont été proposées : par exemple, Beshir et al⁵⁴ ont évalué l'association entre clairance parasitaire après l'administration d'un ACT et transmissibilité aux moustiques par des tests d'alimentation sur membranes (*membrane-feeding assays*). Ils ont ainsi trouvé que les enfants kenyans traités par un protocole standard de 3 jours d'ACT avaient fréquemment une parasitémie résiduelle persistante (mesurée par PCR quantitative), associée à une présence prolongée de gamétocytes dans le sang et une probabilité augmentée

d'infecter les moustiques. Cette approche, bien qu'à la fois innovante et élégante, est également logistiquement contraignante et inadaptée à une application à grande échelle.

Avant 2013, la principale alternative *in vitro* pour l'étude de la résistance à l'ART chez *P. falciparum* était la détermination, en essai classique, des valeurs d' IC_{50} ((i.e., concentration de la drogue qui inhibe 50% de la croissance parasitaire) à la DHA. Cependant, la quasi-totalité des études *in vitro/vivo* (c'est-à-dire toute sauf une⁴⁶) ont rapporté n'avoir trouvé aucune corrélation entre l'issue clinique (demi-vie de la clairance parasitaire) et les valeurs d' IC_{50} à la DHA des isolats^{42, 43}. Une des raisons avancées pour expliquer cette observation réside dans le fait que lors de ces tests, les parasites sont exposés à des doses sub-thérapeutiques de DHA ($\leq 64 \text{ nM}$) pour une durée allant de 48 à 72h, alors qu'*in vivo* les parasites sont exposés à des concentrations bien plus élevées durant 1 à 2h seulement.

C'est avec ces considérations en tête qu'un nouveau test phénotypique *in vitro* a été développé en 2012-2013^{55, 56}. Ce test se base sur l'exposition de parasites adaptés en culture et étroitement synchronisés (format *in vitro*) ou d'isolats frais (format *ex vivo*) à des conditions physiologiquement pertinentes : les parasites sont exposés à 700nM de DHA (le métabolite actif de tous les dérivés d'artémisinine) durant 6 heures, reflétant ainsi la dose type et le temps d'exposition après administration d'un ACT. Après 72h, la susceptibilité des parasites à la drogue est évaluée par microscopie, en estimant la proportion de parasites viables (de morphologie normale) qui ont pu se développer en rings et trophozoites de deuxième génération. Ce test (« Ring-stage Survival Assay », ou RSA) a permis de détecter avec succès des différences significatives entre les parasites provenant de régions avec ou sans résistance clinique avérée. Il a également été constaté que la susceptibilité *in vitro* à la DHA était dépendante du stade de développement du parasite, et fortement associée au stade « jeune ring » (0-3h post-invasion) (Fig. 9). Ceci explique également le manque de

corrélation entre l'issue clinique et les IC₅₀ à la DHA, où les parasites sont exposés tout au long de leur cycle de développement.

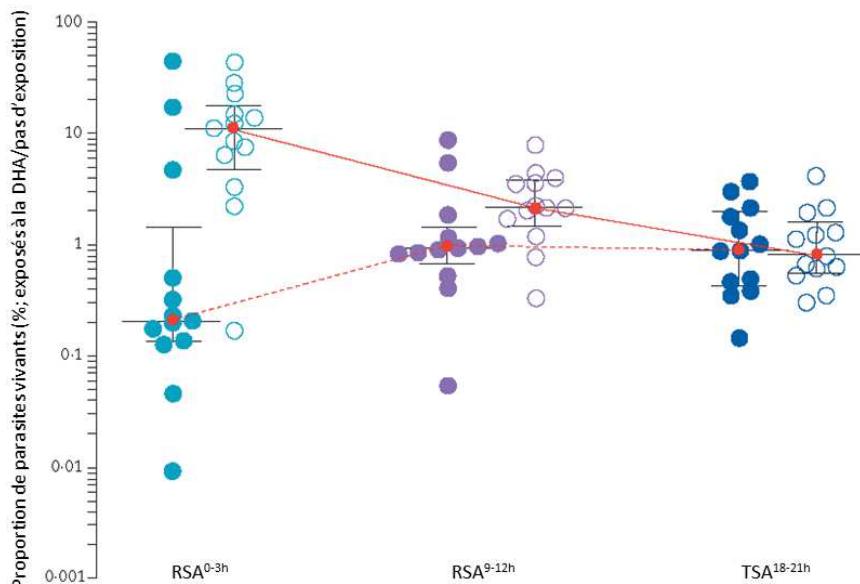


Figure 9. Survie *in vitro* des parasites après exposition à la dihydroartémisinine. Les résultats sont exprimés comme la proportion de parasites survivants après une exposition de 6 heures de parasites âgés de 0 à 3 heures (très jeunes anneaux), âgés de 9 à 12 heures (anneaux âgés) ou de parasites âgés de 18 à 21 heures (trophozoïtes), à 700 nM de dihydroartémisinine (DHA) ou de diméthylsulfoxyde (pour les contrôles).⁵⁷

Ces stades parasitaires sont capables de survivre à une courte exposition à la DHA en arrêtant leur développement de manière transitoire : c'est le phénomène de dormance. Cette observation est en accord avec les découvertes d'études transcriptionnelles antérieures montrant que les parasites ART-résistants avaient un stade ring particulièrement long et une activité métabolique réduite^{58,59}, ainsi qu'avec des études de modélisation ayant suggéré une efficacité moindre des dérivés d'ART contre les parasites au stade ring de l'ouest du Cambodge dès 2007-2008⁶⁰. Les taux de survie obtenus par le RSA étaient également fortement corrélés aux données cliniques⁶¹.

8. Vers la découverte d'un marqueur moléculaire pour la résistance à l'ART ?

Des études préliminaires d'association pangénomique (« genome-wide association study » ou GWAS) ont été menées sur des isolats provenant de régions avec des cas de clairance parasitaire retardée⁶²⁻⁶⁴. Ces études ont permis d'identifier des régions du chromosome 13 associées au phénotype clinique de résistance à l'ART et dénotant d'une sélection positive récente. Une autre information importante a été la découverte de plusieurs sous-populations parasitaires distinctes présentant des niveaux exceptionnels de différentiation génétique mais coexistant dans l'ouest du Cambodge, foyer de la résistance aux antipaludiques. Trois de ces sous-populations (KH2, KH3 et KH4) étaient associées à des délais de clairance parasitaire prolongés⁶⁴ et à des taux de survie élevés en test RSA⁶⁵. Néanmoins, aucun déterminant génétique majeur n'a pu être identifié par ces méthodologies : le test RSA pourrait faciliter la recherche d'un marqueur moléculaire pour la résistance à l'ART.

Il est cependant nécessaire de garder en tête que les dérivés de l'artémisinine sont systématiquement administrés avec une molécule partenaire : il est donc tout aussi important de comprendre les mécanismes impliqués dans l'émergence de parasites résistants aux drogues partenaires, et ce particulièrement dans les zones de résistance à l'ART, où l'élimination des parasites chez les patients dépend essentiellement de l'efficacité de la molécule partenaire.

9. Résistance aux drogues partenaires : le cas de la pipéraquine

La frontière entre le Cambodge et la Thaïlande a toujours été l'épicentre de l'émergence de la résistance aux traitements antipaludiques. Cette région a successivement vu le déclin de la chloroquine (1960s)^{66, 67}, de la sulfadoxine-pyriméthamine (SP) (1970s)^{32, 33} et de la

méfloquine (MQ) (1990s)^{68, 69}. La méfloquine, initialement introduite en monothérapie dans les années 90, fut remplacée en 2000 par l'association artésunate-méfloquine (AS-MQ). L'efficacité clinique de cette combinaison déclina rapidement, de 90,1% en 2001 à 79,4% en 2004, probablement à cause d'une prévalence importante de parasites déjà résistants à la MQ (valeurs élevées d' IC_{50} à la MQ et amplification du nombre de copies du gène Pfmdr1)⁷⁰. L'AS-MQ fut remplacée en 2008 par l'association dihydroartémisinine-pipéraquine (DHA-PPQ), d'abord dans l'ouest du Cambodge (province de Pailin), puis dans l'ensemble du pays en 2010 comme traitement de première intention des accès palustres simples. Les données détaillées des études d'efficacité thérapeutique menées dans différents sites au Cambodge sont présentées dans le tableau 1 et la figure 10.

Tableau 1. Données détaillées des études d'efficacité thérapeutique menées dans différents sites au Cambodge entre 2001 et 2015 afin d'évaluer l'efficacité d'un traitement standard de 3 jours de TCA.

ACPR: adequate clinical and parasitological response (proportion, %); **Suivi:** dernier jour de suivi selon le protocole OMS utilisé (28, 42 or 63 jours); **AS-MQ:** artésunate-méfloquine; **DHA-PPQ:** dihydroartémisinine-pipéraquine; **ATS/PYRO:** artésunate-pyronaridine; **A/L:** artémether-luméfantrine; #: Rapports non-publiés du National Center for Parasitology Entomology and Malaria Control (CNM).

Sites d'étude (Province)	Année de début	Année de fin	Suivi (jours)	TCA utilisé	ACPR (%)	Référence
Battambang	2001	2001	28	AS-MQ	96.0	⁷⁰
	2003	2003	28	AS-MQ	92.3	⁷⁰
	2005	2005	42	AS-MQ	94.1	#
	2007	2007	42	AS-MQ	95.4	#
	2012	2013	42	DHA-PPQ	67.5	⁷¹
	2014	2014	42	ATS/PYRO	100.0	#
Kampong Speu	2003	2003	28	AS-MQ	96.7	⁷⁰
	2010	2010	42	AS-MQ	88.9	⁷²
	2012	2013	42	DHA-PPQ	86.4	⁷¹
Kampong Thom	2012	2012	42	DHA-PPQ	100.0	#
	2012	2013	42	DHA-PPQ	100.0	⁷¹
Kampot	2006	2008	42	AS-MQ	81.2	⁷³
	2013	2014	42	DHA-PPQ	94.1	#
Kratie	2001	2001	28	AS-MQ	100.0	⁷⁰
	2003	2003	28	AS-MQ	100.0	⁷⁰
	2006	2006	42	AS-MQ	97.5	#
	2011	2011	42	DHA-PPQ	96.4	#
	2011	2012	42	DHA-PPQ	83.3	⁷¹
	2013	2013	42	DHA-PPQ	100.0	#
Mondulkiri	2014	2015	42	DHA-PPQ	90.0	#
Odder Meanchey	2003	2003	28	AS-MQ	97.8	⁷⁰
	2012	2013	42	DHA-PPQ	62.5	⁷⁴
	2012	2014	42	DHA-PPQ	51.1	⁷⁵
Pailin	2002	2002	28	AS-MQ	85.7	⁷⁰
	2004	2004	28	AS-MQ	90.1	⁷⁰
	2004	2004	42	AS-MQ	79.3	⁷⁰
	2007	2008	42	AS-MQ	95.0	⁴³
	2007	2008	42	AS-MQ	100.0	⁷⁶
	2007	2008	42	ATS/PYRO	89.8	⁷⁶
	2008	2009	42	DHA-PPQ	89.4	⁷²
	2009	2010	42	DHA-PPQ	91.9	⁷²
	2010	2011	42	DHA-PPQ	75.0	⁷²
	2011	2011	42	AS-MQ	100.0	#
Preah Vihear	2002	2002	28	AS-MQ	96.6	⁷⁰
	2004	2004	28	AS-MQ	100.0	⁷⁰
	2006	2006	42	AS-MQ	98.6	#
	2007	2007	42	AS-MQ	100.0	#

	2009	2009	42	DHA-PPQ	100.0	72
	2009	2009	42	AS-MQ	100.0	#
	2011	2011	42	DHA-PPQ	96.6	#
	2011	2012	42	DHA-PPQ	93.4	71
	2012	2013	63	DHA-PPQ	84.6	78
	2013	2014	42	DHA-PPQ	93.7	#
Pursat	2002	2002	28	AS-MQ	94.0	70
	2004	2004	28	AS-MQ	92.6	70
	2005	2005	42	AS-MQ	87.5	#
	2005	2005	28	A/L	82.4	71
	2005	2005	28	DHA-PPQ	98.2	71
	2007	2007	42	AS-MQ	87.9	#
	2008	2008	42	DHA-PPQ	98.7	72
	2010	2010	42	DHA-PPQ	89.3	72
	2011	2011	42	DHA-PPQ	83.3	#
	2011	2012	42	DHA-PPQ	79.5	71
	2012	2013	63	DHA-PPQ	63.2	78
	2012	2013	42	DHA-PPQ	97.5	71
	2014	2015	42	ATS/PYRO	89.8	77
	2002	2002	28	AS-MQ	100.0	70
Rattanakiri	2004	2004	28	AS-MQ	100.0	70
	2006	2006	42	AS-MQ	96.2	#
	2009	2010	42	DHA-PPQ	100.0	72
	2010	2010	28	A/L	95.0	#
	2010	2010	42	DHA-PPQ	100.0	72
	2010	2010	42	DHA-PPQ	100.0	#
	2012	2013	63	DHA-PPQ	98.4	78
	2013	2013	42	DHA-PPQ	98.3	#
	2014	2014	42	DHA-PPQ	67.6	71
	Siem Reap	2014	2015	DHA-PPQ	37.5	#
Stung Treng	2014	2015	42	DHA-PPQ	65.6	#

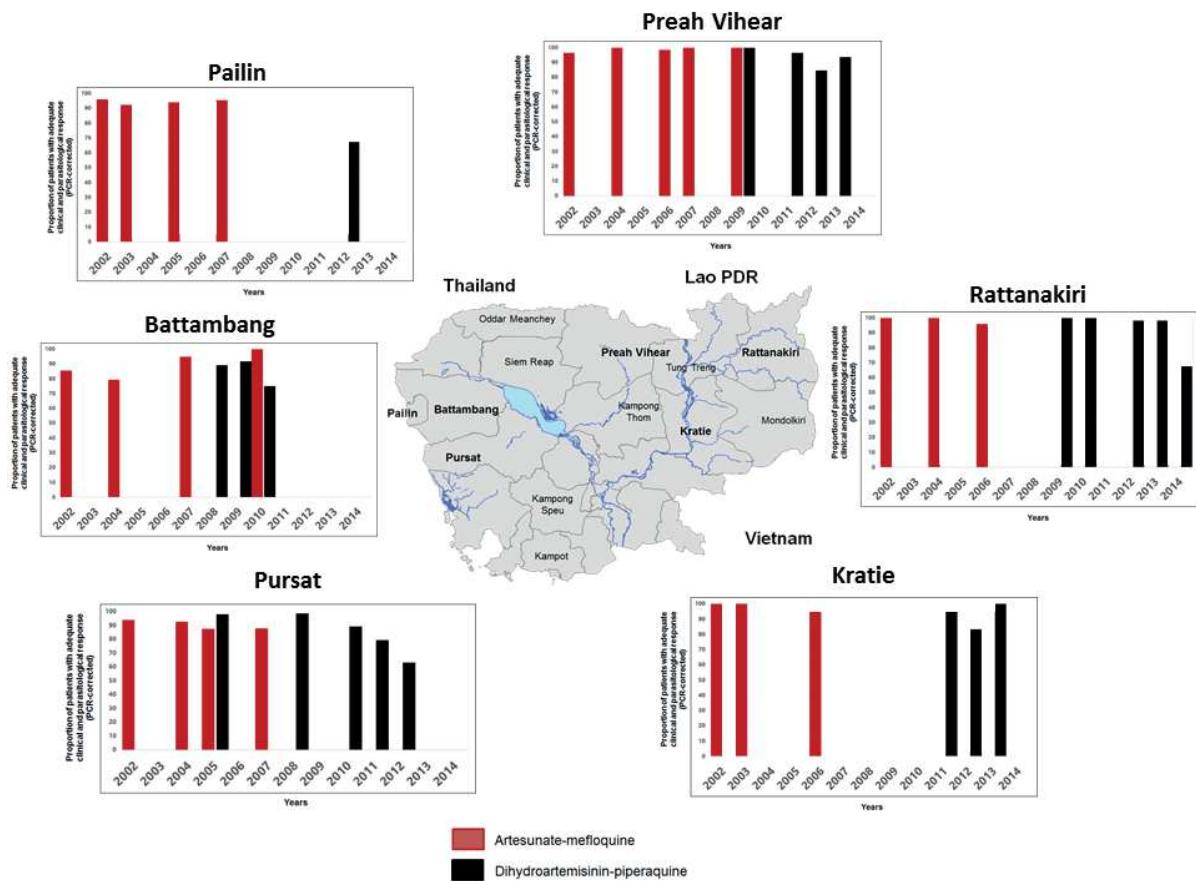


Figure 10. Distribution spatiale et évolution de l'efficacité d'un traitement standard de trois jours d'artésunate-méfloquine ou de ddihydroartémisinine-pipéraquine dans différents sites au Cambodge entre 2001 et 2014.

Ces données illustrent clairement que la baisse d'efficacité des ACTs émerge toujours à l'ouest du Cambodge pour ensuite diffuser à l'est. Il est intéressant de noter que l'association AS-MQ a regagné rapidement son efficacité après son remplacement par l'association DHA-PPQ (par exemple, à Pailin, l'efficacité rapportée de l'AS-MQ en 2004 était d'à peine 79.3% pour ensuite atteindre 100% en 2011, soit trois ans seulement après l'implémentation dans cette région de la DHA-PPQ comme traitement de première intention), suggérant qu'un retour à l'AS-MQ pourrait être une solution temporaire dans les régions où la DHA-PPQ n'est plus efficace.

Enfin, les études d'efficacité de l'association artésunate-pyronaridine (ATS-PYRO) conduites au Cambodge ont révélé des résultats à la fois surprenants et décevants : en effet, l'ATS-

PYRO n'a jamais répondu aux critères d'efficacité de l'OMS pour son utilisation comme traitement de première intention du paludisme à *P. falciparum* à l'ouest du Cambodge, c. à d. une proportion d'ACPR >90% après correction PCR à J42. C'était tout à fait inattendu, étant donné la très bonne efficacité de cet ACT dans d'autres pays endémiques d'Asie et d'Afrique^{76, 79-82}. La raison de cette disparité reste obscure, mais la possibilité d'une résistance croisée entre la pyronaridine et d'autres molécules antipaludiques devrait être étudiée.

Aujourd'hui, l'association DHA-PPQ connaît une baisse drastique d'efficacité au Cambodge. Les études cliniques les plus récentes conduites dans le pays font état d'une augmentation alarmante de la proportion d'échecs de traitement tardifs après un protocole standard de trois jours de DHA-PPQ^{72, 74, 75, 78, 83}. Dans l'ouest du Cambodge, le taux d'échecs de traitement a augmenté de 25% en 2010 à 46% en 2014, indiquant que l'association DHA-PPQ devient inefficace quelques années seulement après son déploiement national. Cela suggère également que les parasites montrant une sensibilité acquise réduite à la fois à l'ART et à la PPQ sont maintenant prévalents au nord-ouest du Cambodge. Si un lien a été établi entre les échecs cliniques à la DHA-PPQ et l'émergence de la résistance à la pipéraquine, aucune corrélation solide n'a été trouvée entre l'issue clinique (recrudescence 2 à 4 semaines après le traitement initial) et la susceptibilité *in vitro* des parasites à la PPQ, évaluée par la détermination de l'IC₅₀ des isolats prétraitement (jour 0). La résistance à la pipéraquine reste mal caractérisée et il n'y a pour le moment aucune preuve rigoureuse de résistance parasitaire à cette molécule (test phénotypique *in vitro* et/ou marqueur moléculaire génétique validé).

Travaux de recherche



**Première Partie. Résistance aux dérivés de l'artémisinine : identification
d'un marqueur moléculaire chez *Plasmodium falciparum***



Chapitre I. Découverte d'un marqueur moléculaire pour la résistance à l'ART chez *P. falciparum*

1. Introduction

L'émergence de *Plasmodium falciparum* résistants aux dérivés d'artémisinine menace les efforts mondiaux récents de contrôle et d'élimination du paludisme. Le risque que de tels parasites diffusent du Cambodge, où ils sont apparus⁴⁶, aux pays alentours puis en Afrique est à la fois réel et inquiétant. La détection précoce et la surveillance continue de la propagation de la résistance à l'ART sont donc essentielles.

Le phénotype clinique de la résistance à l'ART est défini par une clairance parasitaire retardée, ce qui se traduit par une demi-vie de clairance parasitaire augmentée (≥ 5 heures en Asie du Sud-Est)⁴⁹⁻⁵². En 2013, ce paramètre de demi-vie a été corrélé avec les résultats d'un nouveau test phénotypique aux formats *in vitro* et *ex vivo*, le « ring-stage survival assay » ou RSA qui mesure le taux de survie de parasites au stade « jeune ring » à une exposition pharmacologiquement pertinente de DHA (700 nM durant 6 h)^{55, 56}. Cependant, ces méthodes sont logistiquement et financièrement astreignantes et ne sont donc pas adaptées à une détection rapide à grande échelle de parasites résistants à l'ART : un marqueur moléculaire est l'outil indispensable pour une telle utilisation généralisée.

Bien que plusieurs études d'association pangénomique (« genome-wide association study » ou GWAS) aient pu mettre en évidence une sélection positive récente de résistance à l'ART dans certaines régions géographiques⁶²⁻⁶⁴, aucun marqueur moléculaire fiable n'a pour le moment été identifié. Une alternative à de telles études pour la recherche d'un marqueur moléculaire est d'analyser les mutations acquises par une souche de laboratoire adaptée *in vitro* à survivre à de hautes doses d'ART, pour ensuite utiliser ces informations dans l'analyse polymorphe d'isolats cliniques de régions où la résistance à l'ART est bien

documentée. C'est cette stratégie que nous avons utilisée pour étudier les signatures moléculaires de la résistance clinique à l'ART au Cambodge et que nous présentons dans cette publication.

2. Résultats et discussion

La lignée résistante à l'ART F32-ART5 a été sélectionnée par l'équipe de Françoise Benoit-Vical (CNRS Toulouse) en exposant une souche de laboratoire d'origine tanzanienne (F32-Tanzania) à des doses croissantes d'artémisinine pendant cinq ans (125 cycles au total, concentration maximale d'ART de 9 μ M)⁵⁹. La comparaison des exomes de F32-ART5, de sa jumelle F32-TEM (cultivée dans les mêmes conditions mais sans exposition à la drogue) et de la souche parentale F32-Tanzania a mis en évidence huit mutations dans sept gènes. La chronologie de l'apparition de ces mutations a pu être établie par l'analyse du génome de trois clones intermédiaires de la lignée F32-ART, obtenus après 22, 40 et 56 cycles d'exposition. Nous avons par la suite étudié le polymorphisme de ces sept gènes candidats chez 49 isolats cliniques cambodgiens dont nous avions préalablement déterminé le phénotype par le test RSA. Nous avons alors constaté une forte association entre la présence d'une mutation dans le gène *PF3D7_1343700* (dans le domaine en hélice de la protéine kelch K13) et un taux de survie *in vitro* élevé. La présence de parasites mutés a également été corrélée avec une demi-vie de clairance parasitaire augmentée dans les isolats cliniques étudiés par le groupe de Rick Fairhurst (NIAID/NIH, États-Unis). Une étude spatio-temporelle (2001-2012, 6 provinces, 886 échantillons) a montré un envahissement progressif des zones aujourd'hui classées comme résistantes (ouest du Cambodge) par des parasites mutants (18 mutations), tandis que le gène demeurait sauvage dans les régions indemnes (est du Cambodge).

La découverte de ce marqueur moléculaire ouvre la perspective d'une surveillance moléculaire à l'échelle mondiale basée sur la détection de mutations dans le gène *K13*, grâce à des techniques de biologie moléculaire à la fois simples et peu coûteuses. Une telle surveillance permettra de visualiser en temps réel l'aire de distribution des parasites résistants à l'ART et d'adapter les recommandations thérapeutiques en fonction de la situation épidémiologique.

Ce marqueur moléculaire pourrait également nous donner des indications sur les mécanismes cellulaires et moléculaires impliqués dans la résistance à l'ART. En effet, la protéine K13 appartient à la superfamille des protéines kelch, dont le domaine en hélice abrite de multiples sites d'interaction protéine-protéine et intervient dans différentes fonctions cellulaires, telles que la dégradation des protéines ubiquitinylées ou les réponses au stress oxydatif^{84, 85}. D'autres travaux sont nécessaires pour définir la fonction de la protéine K13 et l'effet des différentes mutations ; des études de recombinaison génétique chez des parasites sauvages et mutants pourraient aider à évaluer la contribution des polymorphismes du gène *K13* dans différents fonds génétiques sur les valeurs de survie *in vitro* caractérisées par le test RSA.

3. Article 1

A molecular marker of artemisinin-resistant *Plasmodium falciparum* malaria.

Ariey F, Witkowski B, Amaratunga C, Beghain J, Langlois AC, Khim N, Kim S, Duru V, Bouchier C, Ma L, Lim P, Leang R, Duong S, Sreng S, Suon S, Chuor CM, Bout DM, Ménard S, Rogers WO, Genton B, Fandeur T, Miotto O, Ringwald P, Le Bras J, Berry A, Barale JC, Fairhurst RM, Benoit-Vical F, Mercereau-Puijalon O, Ménard D.

Nature. 2014 Jan 2;505(7481):50-5. doi: 10.1038/nature12876. Epub 2013 Dec 18.

A molecular marker of artemisinin-resistant *Plasmodium falciparum* malaria

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***Plasmodium falciparum* resistance to artemisinin derivatives in southeast Asia threatens malaria control and elimination activities worldwide.** To monitor the spread of artemisinin resistance, a molecular marker is urgently needed. Here, using whole-genome sequencing of an artemisinin-resistant parasite line from Africa and clinical parasite isolates from Cambodia, we associate mutations in the *PF3D7_1343700* kelch propeller domain ('K13-propeller') with artemisinin resistance *in vitro* and *in vivo*. Mutant K13-propeller alleles cluster in Cambodian provinces where resistance is prevalent, and the increasing frequency of a dominant mutant K13-propeller allele correlates with the recent spread of resistance in western Cambodia. Strong correlations between the presence of a mutant allele, *in vitro* parasite survival rates and *in vivo* parasite clearance rates indicate that K13-propeller mutations are important determinants of artemisinin resistance. K13-propeller polymorphism constitutes a useful molecular marker for large-scale surveillance efforts to contain artemisinin resistance in the Greater Mekong Subregion and prevent its global spread.

The emergence of *Plasmodium falciparum* resistance to artemisinin derivatives (ART) in Cambodia threatens the world's malaria control and elimination efforts^{1,2}. The risk of ART-resistant parasites spreading from western Cambodia to the Greater Mekong Subregion and to Africa, as happened previously with chloroquine- and sulphadoxine/pyrimethamine-resistant parasites^{3–5}, is extremely worrisome. Clinical ART resistance is defined as a reduced parasite clearance rate^{1,6–10}, expressed as an increased parasite clearance half-life^{11,12}, or a persistence of microscopically detectable parasites on the third day of artemisinin-based combination therapy (ACT)². The half-life parameter correlates strongly with results from the *in vitro* ring-stage survival assay (RSA_{0–3 h}) and results from the *ex vivo* RSA¹³, which measure the survival rate of young ring-stage parasites to a pharmacologically relevant exposure (700 nM for 6 h) to dihydroartemisinin (DHA)—the major metabolite of all ARTs. However, the present lack of a molecular marker hampers focused containment of ART-resistant parasites in areas where they have been documented and hinders rapid detection of these parasites elsewhere, where ACTs remain the most affordable, effective antimalarials. To detect and monitor the spread of ART resistance, a molecular marker for widespread use is needed.

Recent genome-wide analyses of *P. falciparum* isolates have provided evidence of recent positive selection in geographic areas of ART resistance^{9,14–16}. Whereas parasite heritability of the clinical phenotype

is above 50%, no reliable molecular marker has yet been identified. One possible explanation is that the parasite clearance half-life is not only determined by the intrinsic susceptibility of a parasite isolate to ART, but also by its developmental stage at the time of ART treatment and host-related parameters such as pharmacokinetics and immunity¹⁷. This issue was recently highlighted in patients presenting discordant data between parasite clearance half-life *in vivo* and RSA_{0–3 h} survival rate *in vitro*¹³. Moreover, genome-wide association studies (GWAS) are confounded by uncertainties about parasite population structure. Recent evidence for several highly differentiated subpopulations of ART-resistant parasites in western Cambodia¹⁵ suggests that distinct emergence events might be occurring. An alternative strategy to discover a molecular marker is to analyse mutations acquired specifically by laboratory-adapted parasite clones selected to survive high doses of ART *in vitro*, and use this information to guide analysis of polymorphism in clinical parasite isolates from areas where ART resistance is well documented at both temporal and geographical levels. Here we used this strategy to explore the molecular signatures of clinical ART resistance in Cambodia, where this phenotype was first reported^{1,8}.

A candidate molecular marker of ART resistance

The ART-resistant F32-ART5 parasite line was selected by culturing the ART-sensitive F32-Tanzania clone under a dose-escalating, 125-cycle

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regimen of artemisinin for 5 years¹⁸. Whole-genome sequences were obtained for both F32-ART5 and F32-TEM (its sibling clone cultured without artemisinin) at 460× and 500× average nucleotide coverage, respectively. Compared to F32-TEM, no deleted genes were identified in F32-ART5. The exomes of F32-ART5 and F32-TEM were compared after excluding (1) genes from highly variable, multi-gene families (*var*, *rifin* and *stevor*), (2) positions with coverage lower than 25% of the mean coverage of the parasite line, (3) single-nucleotide polymorphisms (SNPs) found to be mixed in F32-ART5, given that acquired ART-resistance mutation(s) could be expected to be fixed in the sample after 5 years of continuous pressure, (4) SNPs shared between F32-ART5 and the ART-sensitive 3D7 parasite strain and (5) synonymous SNPs (Extended Data Fig. 1).

This analysis identified eight mutations in seven genes that were subsequently confirmed by Sanger sequencing of PCR products (Extended Data Table 1). Each gene harbours one mutant codon in F32-ART5 compared to F32-TEM, F32-Tanzania or 3D7 (Extended Data Table 2). Information on the expression of the genes and the biological function of the proteins are listed in Extended Data Table 3. Only one of these genes, cysteine protease falcipain 2a (*PF3D7_1115700*), has previously been associated with *in vitro* responses to ART¹⁹. To determine when each mutation arose in the F32-ART5 lineage, we analysed the whole-genome sequences of parasites at various drug-pressure cycles (Fig. 1). This analysis showed that the *PF3D7_0110400* D56V and *PF3D7_1343700* M476I mutations were acquired first, during the steep increase of ART resistance, and remained stable thereafter. Importantly, the appearance of these two mutations is associated with an increase in the RSA_{0–3 h} survival rate, from less than 0.01% to 12.8%. Subsequent PCR analysis of the *PF3D7_1343700* locus detected the M476I mutation after 30 drug-pressure cycles, consistent with the sharp increase in RSA_{0–3 h} survival rate observed thereafter. The other SNPs appeared stepwise at later stages of selection: *PF3D7_0213400* (68 cycles); *PF3D7_1115700* (98 cycles); *PF3D7_1302100*, *PF3D7_1459600* and *PF3D7_1464500* (120 cycles) (Extended Data Table 2). These data indicate that the *PF3D7_1343700* M476I mutation increased the resistance of F32-Tanzania to DHA in the RSA_{0–3 h}.

To explore whether these mutations are associated with ART resistance in Cambodia, we investigated sequence polymorphism in all seven genes by mining whole-genome or Sanger sequences for 49 culture-adapted parasite isolates collected in 2010–2011 (see Methods). We chose these isolates based on their differential RSA_{0–3 h} survival rates (Supplementary Table 1) and their sequences were compared to those of control parasite lines 3D7, 89F5²⁰ and K1992 (see Methods). Three genes (*PF3D7_0110400*, *PF3D7_0213400* and *PF3D7_1302100*) encode a wild-type sequence for all parasite isolates. The other four genes show intra-population diversity, with previously reported or novel SNPs (Supplementary Table 1). *PF3D7_1115700* has 11 SNPs that are not associated with RSA_{0–3 h} survival rates ($P = 0.06$, Kruskal–Wallis test). *PF3D7_1459600* has 6 SNPs that are not associated with survival rates ($P = 0.65$). *PF3D7_1464500* has 12 SNPs previously reported in older isolates from southeast Asia, including the ART-susceptible Dd2 line²¹, probably reflecting a geographic signature. These SNPs also show no significant association with survival rates ($P = 0.42$). Therefore, these six genes were not studied further.

In contrast, *PF3D7_1343700* polymorphism shows a significant association with RSA_{0–3 h} survival rates (Fig. 2). Indeed, RSA_{0–3 h} survival rates differ substantially between parasite isolates with wild-type (median 0.17%, range 0.06–0.51%, $n = 16$) or mutant (18.8%, 3.8–58%, $n = 33$) K13-propeller alleles ($P < 10^{-4}$, Mann–Whitney *U* test) (Supplementary Table 1). Four mutant alleles are observed, each harbouring a single non-synonymous SNP within a kelch repeat of the C-terminal K13-propeller domain, namely Y493H, R539T, I543T and C580Y located within repeats no. 2, 3, 3 and 4, respectively. Both the K1992 and the ART-susceptible 89F5 lines carry a wild-type K13-propeller. There are no associations between polymorphisms in the K13-propeller and those in the other candidate genes (Supplementary Table 1). Based on these

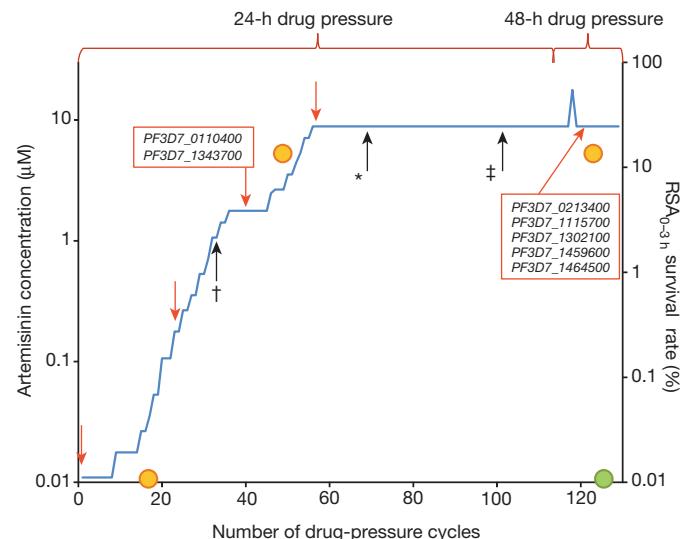


Figure 1 | Temporal acquisition of mutations in F32-ART5. F32-Tanzania parasites exposed to increasing artemisinin concentrations for 120 consecutive cycles¹⁸ were analysed by whole-genome sequencing at five time-points (red arrows). Loci mutated after a given number of drug-pressure cycles are shown (red boxes). The earliest time-points where three mutations were detected by PCR (black arrows) are indicated by † for *PF3D7_1343700*, * for *PF3D7_0213400* and ‡ for *PF3D7_1115700*. Orange and green circles indicate RSA_{0–3 h} survival rates for F32-ART5 and F32-TEM parasites, respectively (mean of 3 experiments each).

observations and the acquisition of M476I in kelch repeat no. 2 by F32-ART5, we investigated whether K13-propeller polymorphism is a molecular signature of ART resistance in Cambodia.

Emergence and spread of K13-propeller mutants in Cambodia

Over the last decade, the prevalence of ART resistance has steadily increased in the western provinces of Cambodia, but not elsewhere in

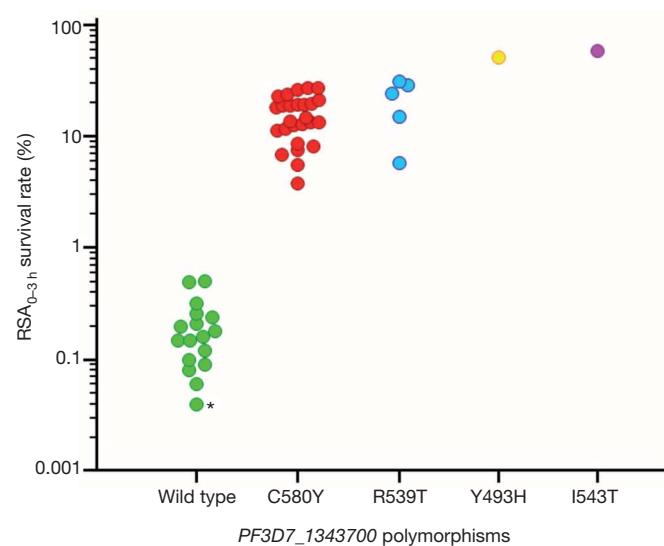


Figure 2 | Survival rates of Cambodian parasite isolates in the RSA_{0–3 h}, stratified by K13-propeller allele. Genotypes were obtained by mining whole-genome sequence data ($n = 21$) or sequencing PCR products ($n = 28$). Mutant parasites have significantly higher RSA_{0–3 h} survival rates than wild-type parasites: wild type ($n = 17$, median 0.16%, IQR 0.09–0.24, range 0.04–0.51); C580Y ($n = 26$, median 14.1%, IQR 11.3–19.6, range 3.8–27.3, $P < 10^{-6}$ for wild type versus C580Y, Mann–Whitney *U* test); R539T ($n = 5$, median 24.2%, IQR 12.6–29.5, range 5.8–31.3, $P < 10^{-3}$ for wild type versus R539T); Y493H (51.4%); and I543T (58.0%). The RSA_{0–3 h} survival rate (0.04%) of control 3D7 parasites is indicated by an asterisk.

the country². To test whether the spatiotemporal distribution of K13-propeller mutations correlates with that of ART resistance, we sequenced the K13-propeller of archived parasite isolates from Cambodian patients with malaria in 2001–2012 (Extended Data Table 4). Data from six provinces were compared ($n = 886$): Pailin, Battambang and Pursat in the west where ART resistance is established^{1,6,8,22}, Kratie in the southeast where ART resistance has increased in recent years², and Preah Vihear in the north and Ratanakiri in the northeast where there was virtually no evidence of ART resistance during this time period². This analysis reveals overall 17 mutant alleles, including three high-frequency ($> 5\%$) alleles (C580Y, R539T and Y493H). The frequency of wild-type sequence decreased significantly over time in all three western provinces, but not in Preah Vihear or Ratanakiri. The frequency of the C580Y allele increased significantly from 2001–2002 to 2011–2012 in Pailin and Battambang, indicating its rapid invasion of the population and near fixation in these areas (Fig. 3).

To further investigate the geographic diversity of K13-propeller polymorphism in Cambodia, we extended our sequence analysis to include data from four additional provinces ($n = 55$, Kampong Som, Kampot, Mondulkiri and Oddar Meancheay) in 2011–2012 (Extended Data Table 4). Although a large number of mutations are observed (Supplementary Fig. 1 and Extended Data Table 5), the C580Y allele accounts for 85% (189/222) of all mutant alleles observed in 2011–2012 (Extended Data Fig. 2). This mapping outlines the elevated frequency (74%, 222/300) of parasites harbouring a single non-synonymous mutation in the K13-propeller and the geographic disparity of their distribution. Importantly, the frequency distribution of mutant alleles over the various provinces matches that of day 3 positivity in patients treated for malaria with an ACT (Spearman's $r = 0.99$, 95% confidence interval 0.96–0.99, $P < 0.0001$), considered a suggestive sign of clinical ART resistance (Extended Data Fig. 3).

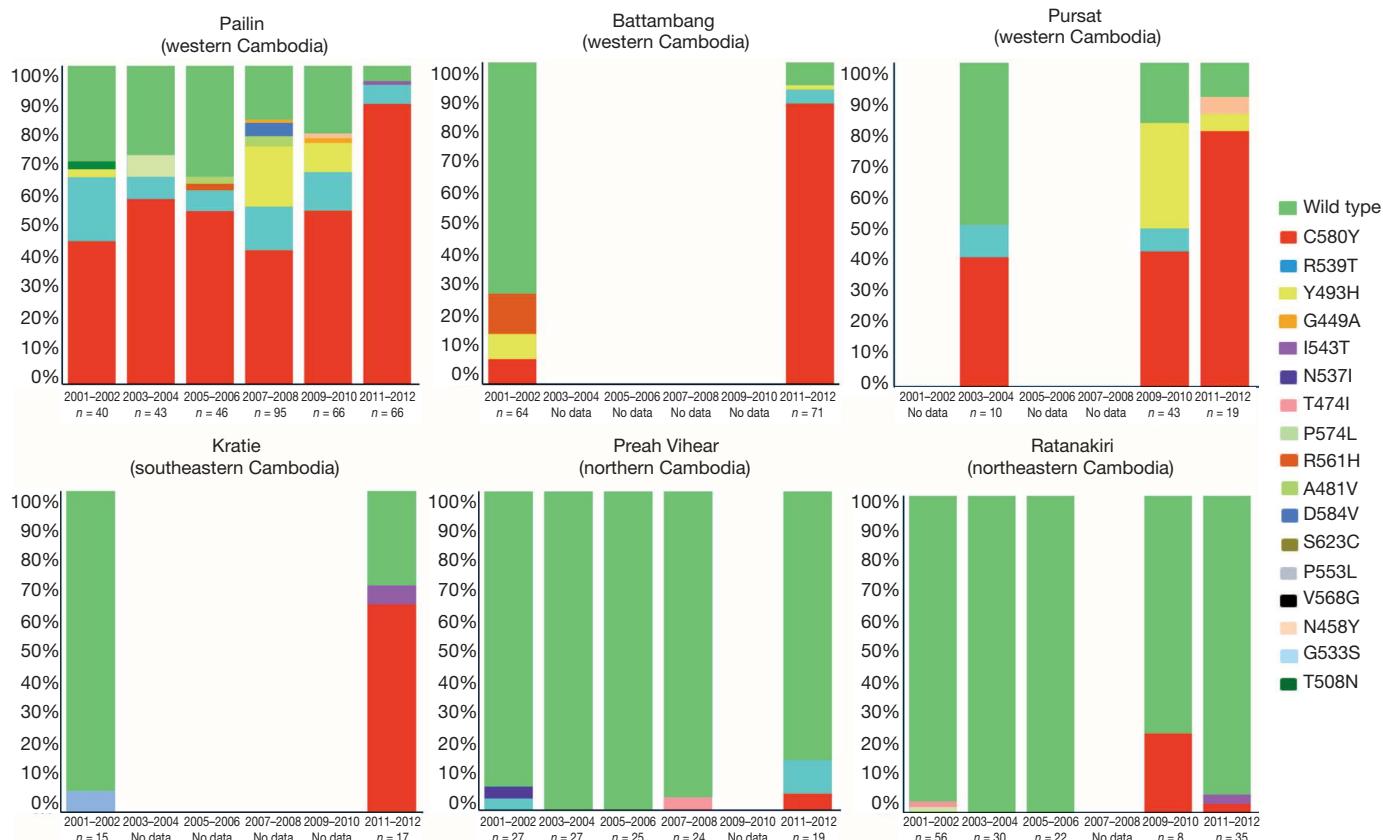


Figure 3 | Frequency of K13-propeller alleles in 886 parasite isolates in six Cambodian provinces in 2001–2012. Genotypes were obtained by sequencing PCR products from archived blood samples. All mutant alleles carry a single non-synonymous SNP (colour-coded, same colour codes as in Fig. 2 for wild type, C580Y, R539T, Y493H and I543T). Significant reductions (Fisher's exact test) in wild-type allele frequencies were observed in Pailin, Battambang, Pursat and Kratie over time (see Methods).

K13-propeller polymorphisms and clinical ART resistance

To confirm that K13-propeller polymorphism is a molecular marker of clinical ART resistance, we first identified 163 patients from Pursat and Ratanakiri in whom we measured parasite clearance half-lives (range 1.58–11.53 h)⁶ in 2009–2010 and for which parasites were previously assigned to a KH subpopulation (KH1, KH2, KH3, KH4 or KHA) on the basis of ancestry analysis of whole-genome sequence data¹⁵. Thirteen patients with mixed genotypes (a wild-type and one or more mutant K13-propeller alleles) were excluded. Of the remaining 150 patients, 72 carried parasites with a wild-type allele and the others carried parasites with only a single non-synonymous SNP in the K13-propeller: C580Y ($n = 51$), R539T ($n = 6$) and Y493H ($n = 21$) (Extended Data Table 6). The parasite clearance half-life in patients with wild-type parasites is significantly shorter (median 3.30 h, interquartile range (IQR) 2.59–3.95) than those with C580Y (7.19 h, 6.47–8.31, $P < 10^{-6}$, Mann–Whitney U test), R539T (6.64 h, 6.00–6.72, $P < 10^{-4}$) or Y493H (6.28 h, 5.37–7.14, $P < 10^{-6}$) parasites (Fig. 4a). Also, the parasite clearance half-life in patients carrying C580Y parasites is significantly longer than those with Y493H parasites ($P = 0.007$, Mann–Whitney U test). These data indicate that C580Y, R539T and Y493H identify slow-clearing parasites in malaria patients treated with ART.

Because KH2, KH3, KH4 and KHA parasites have longer half-lives than KH1 parasites¹⁵, we proposed that allelic variation in the K13-propeller accounts for these differences. Among 150 parasites, 55, 26, 14, 12 and 43 are classified as KH1, KH2, KH3, KH4 and KHA, respectively. Three K13-propeller alleles strongly associate with KH groups: 96% (53/55) of KH1, 96% (25/26) of KH2 and 100% (12/12) of KH4 parasites carry the wild-type, C580Y and Y493H alleles, respectively (Extended Data Table 6). Whereas KH3 parasites ($n = 14$) carry the wild-type, C580Y and R539T alleles, R539T is not observed in KH1, KH2 or KH4

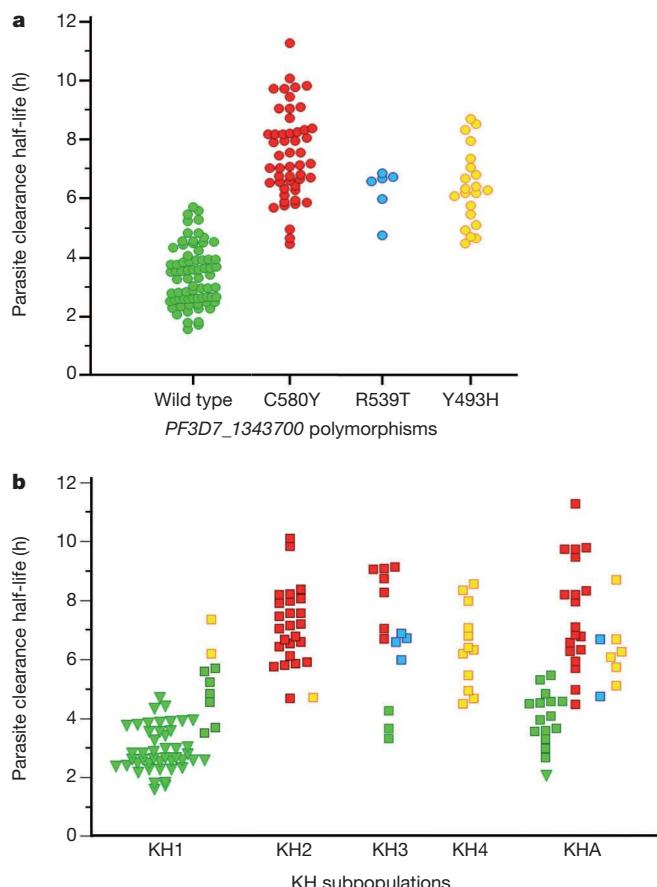


Figure 4 | Parasite clearance half-lives. **a**, Correlation of parasite clearance half-lives and K13-propeller alleles for parasite isolates in Pursat and Ratanakiri in 2009–2010. Wild-type parasites have shorter half-lives (median 3.30 h, IQR 2.59–3.95, $n = 72$) than C580Y (7.19 h, 6.47–8.31, $n = 51$, $P < 10^{-6}$, Mann–Whitney U test), R539T (6.64 h, 6.00–6.72, $n = 6$, $P < 10^{-6}$) or Y493H (6.28 h, 5.37–7.14, $n = 21$, $P < 10^{-6}$) parasites. The half-life of C580Y parasites is significantly longer than that of Y493H parasites ($P = 0.007$). **b**, Correlation of parasite clearance half-lives, KH subpopulations¹⁵ and K13-propeller alleles for the same 150 parasite isolates. Half-lives are shown for Pursat (squares) and Ratanakiri (triangles) parasites, stratified by KH group and K13-propeller allele (colour-coded as in **a**). Median half-lives stratified by K13-propeller allele are KH1: wild type (2.88) and Y493H (6.77); KH2: C580Y (7.13) and Y493H (4.71); KH3: wild type (3.65), C580Y (8.73) and R539T (6.65); KH4: Y493H (6.37); and KHA: wild type (4.01), C580Y (7.09), Y493H (6.18) and R539T (5.73).

parasites. As expected, KHA parasites have a mixed allele composition. Importantly, K13-propeller mutations more accurately identify slow-clearing parasites than KH group (Fig. 4b), demonstrating that the association of K13-propeller polymorphism with clinical ART resistance in Cambodia is partially independent of the genetic background of KH subpopulations. Within the KH1 group ($n = 55$), the parasite clearance half-life in patients with wild-type parasites is significantly shorter ($n = 53$, median 2.88 h, IQR 2.52–3.79) than those with Y493H parasites ($n = 2$, median 6.77 h, $P = 0.02$, Mann–Whitney U test). Within the KH3 subpopulation ($n = 14$), the half-life in patients with wild-type parasites is shorter ($n = 3$, median 3.65 h) than those with C580Y ($n = 7$, median 8.73 h, IQR 7.35–9.06, $P = 0.02$) or R539T ($n = 4$, 6.65 h, 6.29–6.80, $P = 0.03$) parasites.

Discussion

The F32-ART5 lineage acquired a K13-propeller mutation as it developed ART resistance, as indicated by its ability to survive a pharmacologically relevant exposure to DHA in the RSA_{0–3 h}. Genes putatively associated with ART resistance (*Pfcrt*^{23,24}, *Pftcp*^{25,26}, *Pfmdr1*^{8,27,28}, *Pfnmrp1*^{27–29} and

ABC transporters³⁰) or encoding putative targets of ART (*PfATPase6*^{31,32} and *Pfubc6*—the orthologue of *Plasmodium chabaudi ubp1*^{33,34}) were not mutated during the 5-year selection of F32-ART5, and *Pfmdr1* amplification was not observed^{35–40}. In addition, all candidate ART-resistance genes recently identified using population genetics approaches^{14,40,41} remained unaltered in F32-ART5, except for *PF3D7_1343700* and *PF3D7_1459600* located in the linkage-disequilibrium windows identified in ref. 16. These findings led us to identify another 17 single K13-propeller mutations in naturally circulating parasites in Cambodia. Several of these mutations associate strongly with the spatiotemporal distribution of ART resistance in Cambodia, increased parasite survival rates in response to DHA *in vitro*, and long parasite clearance half-lives in response to ART treatment *in vivo*. None of the six other genes mutated in F32-ART5 associate with RSA_{0–3 h} survival rates in parasite isolates from Cambodia.

K13-propeller polymorphism fulfils the definition of a molecular marker of ART resistance for several reasons: (1) there has been a progressive loss of wild-type parasites in western Cambodia during the decade of emerging ART resistance in this region; (2) mutant parasites cluster in Cambodian provinces where ART resistance is well established and are less prevalent where ART resistance is uncommon; (3) *PF3D7_1343700* is located 5.9 kilobases upstream of the 35-kb locus identified in ref. 14 as being under recent positive selection, and within the region of top-ranked signatures of selection outlined in ref. 16; (4) multiple mutations, all non-synonymous, are present in the K13-propeller, reflecting positive selection rather than a hitchhiking effect or genetic drift; (5) mutations occur in a domain that is highly conserved in *P. falciparum*, with only one non-synonymous SNP being documented in a single parasite isolate from Africa⁴²; (6) all polymorphisms we observe in Cambodia are novel and all but one (V568G) occur at positions strictly conserved between *Plasmodium* species (Supplementary Fig. 1 and Supplementary Fig. 2), suggesting strong structural and functional constraints on the protein; (7) the three most-prevalent K13-propeller mutations correlate strongly with RSA_{0–3 h} survival rates *in vitro* and parasite clearance half-lives *in vivo* at the level of individual parasite isolates and malaria patients, respectively; and (8) the frequency of mutant alleles correlates strongly with the prevalence of day 3 positivity after ACT treatment at the level of human populations in Cambodia.

On the basis of homology with other kelch propeller domains, we anticipate that the observed K13-propeller mutations destabilize the domain scaffold and alter its function. The carboxy-terminal portion of *PF3D7_1343700* encodes six kelch motifs, which are found in a large number of proteins with diverse cellular functions^{43,44}. Given that the toxicity of ART derivatives depends principally on their pro-oxidant activity, the reported role of some kelch-containing proteins in regulating cytoprotective and protein degradation responses to external stress is particularly intriguing. The K13-propeller shows homology with human KLHL12 and KLHL2, involved in ubiquitin-based protein degradation, and KEAP1, involved in cell adaptation to oxidative stress (Extended Data Fig. 4). More work is needed to delineate the normal function of K13 and the effect of various mutations. Allele exchange studies in mutant and wild-type parasites may help to define the contribution of K13-propeller polymorphisms on different genetic backgrounds to the RSA_{0–3 h} survival rate. Indeed, it is particularly worrying that as few as two mutations, that is, the K13-propeller M476I and *PF3D7_0110400* D56V, were sufficient to confer ART resistance to F32-Tanzania, which has a typical African genetic background. Cambodian parasites with mutant K13-propellers display a wide range of RSA_{0–3 h} survival rates (3.8–58%) and parasite clearance half-lives (4.5–11.5 h). Further studies are therefore required to identify additional genetic determinants of ART resistance, which may reside in the strongly selected regions recently identified^{14,16}. In this context, analysing the RSA_{0–3 h} survival rates as a quantitative trait among parasites harbouring the same K13-propeller mutation could help to identify additional genetic loci involved in ART resistance.

In summary, K13-propeller polymorphism seems to be a useful molecular marker for tracking the emergence and spread of ART-resistant *P. falciparum*.

METHODS SUMMARY

The ART-resistant F32-ART5 parasite line was selected by culturing the ART-sensitive F32-Tanzania clone under a dose-escalating regimen of artemisinin for 5 years. The F32-TEM line was obtained by culturing F32-Tanzania in parallel without artemisinin exposure. Reference DNA was extracted from *P. falciparum* lines 3D7, 89F5 Palo Alto Uganda and K1992. The ring-stage survival assay (RSA_{0-3h}) was performed as described previously¹³. Whole-genome sequencing was performed on F32-Tanzania, F32-TEM, F32-ART5 (4 time points), three reference strains (3D7, 89F5 and K1992) and 21 Cambodian parasite isolates, using an Illumina paired-reads sequencing technology. A set of 1091 clinical *P. falciparum* isolates was collected from patients participating in ACT efficacy studies in 2001–2012. The K13-propeller was amplified using nested PCR. Double-strand sequencing of PCR products was performed by Macrogen. Sequences were analysed with MEGA 5 software version 5.10 to identify specific SNP combinations. Data were analysed with Microsoft Excel and MedCalc version 12. Differences were considered statistically significant when *P* values were less than 0.05. Ethical clearances for parasite isolate collections were obtained from the Cambodian National Ethics Committee for Health Research, the Institutional Review Board of the Naval Medical Research Center, the Technical Review Group of the WHO Regional Office for the Western Pacific, and the Institutional Review Board of the National Institute of Allergy and Infectious Diseases.

Online Content Any additional Methods, Extended Data display items and Source Data are available in the online version of the paper; references unique to these sections appear only in the online paper.

Received 15 July; accepted 12 November 2013.

Published online 18 December 2013.

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Supplementary Information is available in the online version of the paper.

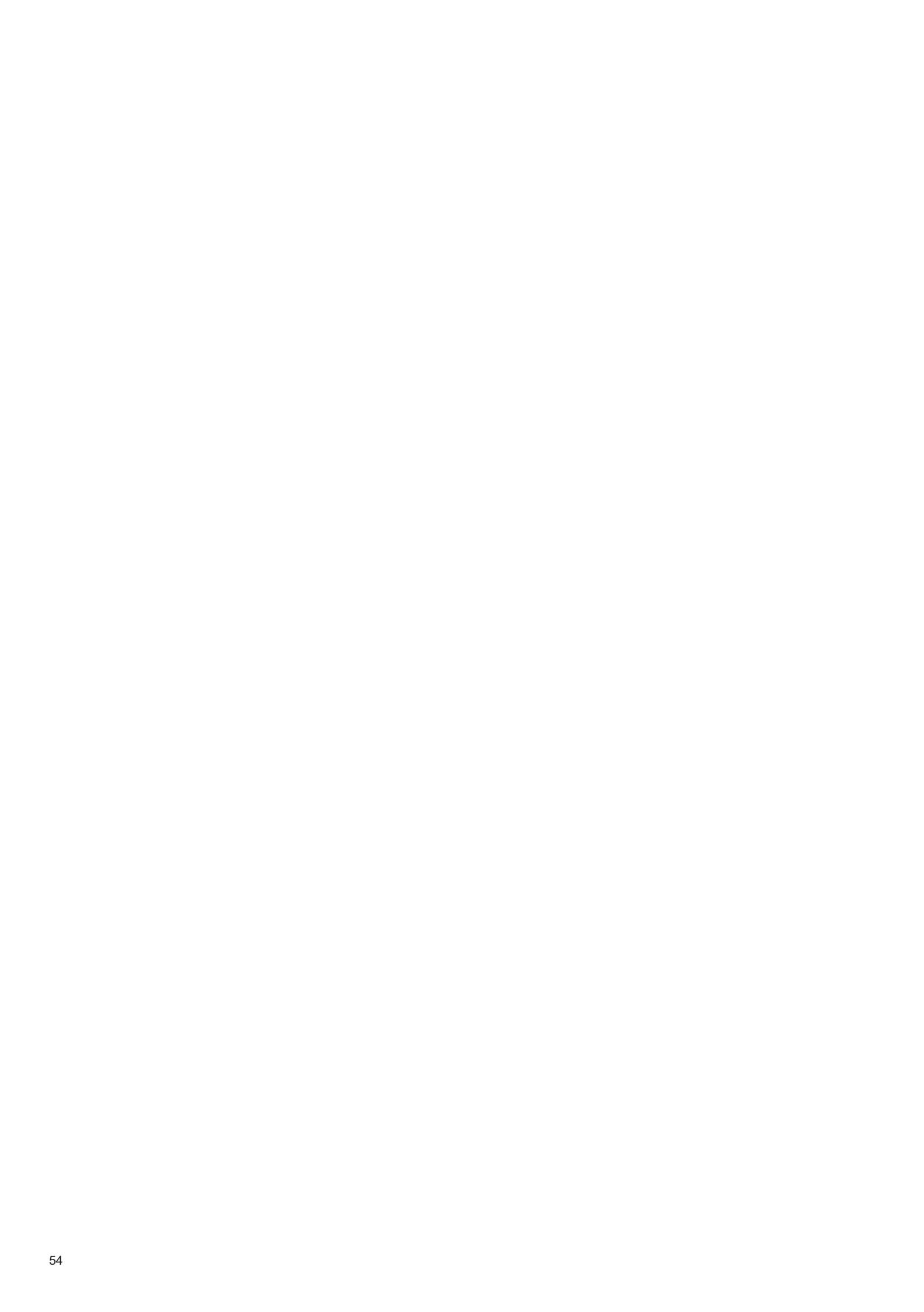
Acknowledgements We thank the patients and field staff involved in clinical trials and sample collections. We are grateful to the provincial health department directors and other staff of the Cambodian Ministry of Health. Clinical trials and sample collections were supported in part by the Global Fund Grant Malaria Program Rounds 6 (CAM-607-G10M-CNM3) and 9 (CAM-S10-G14-M), the Bill and Melinda Gates Foundation and USAID (through the World Health Organization), the US DOD Global Epidemic Information System, and the Intramural Research Program, NIAID, NIH. Laboratory work was supported by grants from Banque Natixis (to O.M.-P. and D.M.) and Laboratoire d'Excellence IBEID (Agence Nationale de la Recherche, France) and Institut Pasteur, Division International (ACIP A-10-2010). B.W. was supported by a postdoctoral fellowship from Institut Pasteur, Division International; J.B. by an Institut Pasteur Paris Master-Pro fellowship; and D.M. by the French Ministry of Foreign Affairs. We are grateful to the Wellcome Trust Sanger Institute and the MalariaGEN resource centre for sequencing, genotyping and population structure analysis of some Cambodian clinical samples, funded by the Wellcome Trust (098051; 090770/Z/

09/Z) and the MRC (G0600718). We thank the Rotary Club-Versailles for funding computer equipment. P.R., D.M.B. and W.O.R. are staff members of the World Health Organization and the US Navy, respectively. They alone are responsible for the views expressed in this publication, and they do not necessarily represent the decisions, policy or views of the World Health Organization or the US Navy.

Author Contributions B.W., S.M., A.B. and F.B.-V. produced the F32-ART5 and F32-TEM clonal lines and analysed their survival rates. F.A. and J.B. developed computational components of the whole-genome sequence analysis. C.B. and L.M. performed whole-genome sequencing. F.A., C.A., S.K., V.D., P.L., R.L., S.D., Se.S., So.S., C.M.C., D.M.B., W.O.R., B.G., T.F., P.R., J.L.B., R.M.F. and D.M. conducted clinical studies and collected parasite isolates. A.-C.L., N.K., S.K., V.D., S.M. and A.B. performed PCR and sequencing analyses. B.W., F.B.-V., V.D. and D.M. performed *in vitro* assays (RSA₀₋₃₁). O.M. provided genotyping and population structure data for Cambodian parasite isolates. J.-C.B. and O.M.-P. performed three-dimensional structure modelling. F.A., R.M.F., F.B.-V., O.M.-P.

and D.M. conceived of the study, supervised the project, processed the data and wrote the manuscript with contributions from B.W., C.A., A.B. and J.-C.B.

Author Information The following reagents have been deposited to the MR4/BEI by D.M.: MRA-1236 (*Plasmodium falciparum* IPC 3445 Pailin Cambodia 2010), MRA-1237 (*Plasmodium falciparum* IPC 3663 Pailin Cambodia 2010), MRA-1238 (*Plasmodium falciparum* IPC 4884 Pursat Cambodia 2011), MRA-1239 (*Plasmodium falciparum* IPC 5188 Ratanakiri Cambodia 2011), MRA-1240 (*Plasmodium falciparum* IPC 5202 Battambang Cambodia 2011) and MRA-1241 (*Plasmodium falciparum* IPC 4912 Mondulkiri Cambodia 2011). Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of the paper. Correspondence and requests for materials should be addressed to F.A. (frederic.ariey@pasteur.fr), O.M.-P. (odile.puijalon@pasteur.fr), D.M. (dmenard@pasteur-kh.org), F.B.-V. (Francoise.Vical@inserm.fr) and R.M.F. (rfairhurst@niaid.nih.gov).



Chapitre II. Les mutations dans le domaine en hélice du gène *K13* confèrent la résistance à l'ART chez des isolats cliniques de *P. falciparum*

1. Introduction

Comme présenté dans l'article précédent, la présence de mutations dans le domaine en hélice du gène *K13* a été identifiée comme marqueur moléculaire candidat de la résistance à l'ART⁸⁶. Ce gène se situe sur le chromosome 13 de *P. falciparum*, près de régions précédemment associées au phénotype clinique de clairance parasitaire retardée⁶²⁻⁶⁴. L'analyse génomique d'isolats cliniques du Cambodge a identifié quatre mutations prévalentes (Y493H, R539T, I543T et C580Y), associées à des taux de survie *in vitro* élevés (évalués par test RSA) et des demi-vies d'élimination parasitaire >5 heures. Il est maintenant nécessaire de déterminer si les mutations du gène *K13* confèrent la résistance à l'ART dans des isolats cliniques et d'évaluer la contribution de chaque polymorphisme dans différents fonds génétiques, et ce afin d'élucider à terme les mécanismes moléculaires impliqués.

Pour ce faire, nous avons développé des nucléases à doigt de zinc (ou *zinc-finger nuclease*, ZFN) permettant la recombinaison génétique ciblée du gène *K13*. Les ZFNs sont des enzymes de restriction artificielles créées par l'association d'un domaine spécifique de liaison à l'ADN (type *zinc-finger*) et d'un domaine catalytique de coupure de l'ADN (nucléase). Les ZFNs sont introduites dans les formes intra-érythrocytaires du parasite par électroporation, en même temps que des plasmides donneurs contenant une certaine version du gène *K13*. Les ZFNs déclenchent alors une cassure double brin au niveau du locus du gène *K13*, menant à la réparation de l'ADN par capture de la version du gène portée par le plasmide donneur⁸⁷.

Cette stratégie nous a permis d'introduire ou de retirer des mutations dans un ensemble d'isolats cliniques cambodgiens, ainsi que dans des lignées de référence de différentes origines géographiques.

2. Résultats et discussion

À l'aide de plasmides donneurs contenant la version sauvage du gène *K13*, nous avons retiré les mutations individuelles de plusieurs isolats cambodgiens résistants à l'ART. Les taux de survie *in vitro*, qui étaient compris entre 13 et 49% chez les souches parentales, ont alors diminué entre 0.3 et 2.4%. À l'inverse, l'introduction d'une mutation du gène *K13* dans des parasites sauvages (isolats cliniques à clairance rapide ou lignées de référence) a entraîné une nette augmentation des taux de survie *in vitro*, de $\leq 0.6\%$ à des valeurs comprises entre 2 et 29%. Ces données démontrent bien un rôle central des mutations dans le domaine en hélice du gène *K13* dans la résistance à l'ART chez *P. falciparum*.

Nous avons ensuite examiné si les mutations individuelles conféraient différents degrés de résistance à l'ART lorsqu'insérées tour à tour dans un même fond génétique. Dans la lignée de référence DD2, l'introduction des mutations M476L (mutation identifiée dans la souche F32-ART), R539T ou I543T a conféré des degrés de résistance bien supérieurs que celle des mutations Y493H ou C580Y (10 à 30% contre 2 à 4%, respectivement). Ce résultat est assez surprenant, la mutation C580Y étant rapidement devenue l'allèle muté prédominant à l'ouest du Cambodge. Nous avons donc étudié l'impact de l'introduction de la mutation C580Y dans différents fonds génétiques : il s'est avéré que cette mutation conférait un degré de résistance plus important dans les isolats cambodgiens que dans les lignées de référence DD2 et FCB, suggérant que des facteurs parasitaires additionnels jouent un rôle important dans la modulation de la résistance à l'ART chez ces isolats cliniques contemporains. Le contraste entre le degré de résistance *in vitro* relativement faible conféré par C580Y et sa large dissémination au Cambodge pourrait s'expliquer par un coût de fitness moindre ou un potentiel de transmission augmenté chez les parasites porteurs de cet allèle, ou encore par un fond génétique particulier du parasite⁸⁸.

Cette étude a permis de valider le rôle central joué par les mutations dans le domaine en hélice du gène *K13* et justifie ainsi la nécessité de cartographier la présence ou l'absence de parasites mutants à l'échelle planétaire, et ce afin de surveiller la dissémination de la résistance à l'ART et de limiter son impact sur les programmes nationaux de lutte et de contrôle du paludisme. Cette technique d'édition génomique pourra également être réutilisée dans le but d'analyser de potentielles nouvelles mutations et d'identifier celles qui confèrent la résistance à l'ART.

3. Article 2

Drug resistance. K13-propeller mutations confer artemisinin resistance in Plasmodium falciparum clinical isolates.

Straimer J, Gnädig NF, Witkowski B, Amaratunga C, Duru V, Ramadani AP, Dacheux M, Khim N, Zhang L, Lam S, Gregory PD, Urnov FD, Mercereau-Puijalon O, Benoit-Vical F, Fairhurst RM, Ménard D, Fidock DA.

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ACKNOWLEDGMENTS

We thank S. Kwon and V. Zyryanov for help with EXAFS measurements and N. James for reading the manuscript. The work was supported by the II-VI Foundation, U.S. Department of

Energy (DOE) SunShot program under award DE-EE0005312 and by NSF under award DMR-1310398. D.V.T. also thanks the Keck Foundation. This work used facilities supported by the NSF Materials Research Science and Engineering Center Program under awards DMR 08-20054 and DMR 14-20709. Use of the Center for Nanoscale Materials and Advanced Photon Source was supported by the DOE, Office of Science, Office of Basic Energy Sciences, under contract DE-AC02-06CH11357. MRCAT operations are supported by the DOE and MRCAT host institutions.

SUPPORTING ONLINE MATERIAL

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Materials and Methods
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27 August 2014; accepted 8 December 2014
10.1126/science.1260501

DRUG RESISTANCE

K13-propeller mutations confer artemisinin resistance in *Plasmodium falciparum* clinical isolates

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The emergence of artemisinin resistance in Southeast Asia imperils efforts to reduce the global malaria burden. We genetically modified the *Plasmodium falciparum* K13 locus using zinc-finger nucleases and measured ring-stage survival rates after drug exposure *in vitro*; these rates correlate with parasite clearance half-lives in artemisinin-treated patients. With isolates from Cambodia, where resistance first emerged, survival rates decreased from 13 to 49% to 0.3 to 2.4% after the removal of K13 mutations. Conversely, survival rates in wild-type parasites increased from ≤0.6% to 2 to 29% after the insertion of K13 mutations. These mutations conferred elevated resistance to recent Cambodian isolates compared with that of reference lines, suggesting a contemporary contribution of additional genetic factors. Our data provide a conclusive rationale for worldwide K13-propeller sequencing to identify and eliminate artemisinin-resistant parasites.

The worldwide use of artemisinin (ART)-based combination therapies (ACTs) for the treatment of *Plasmodium falciparum* malaria is the foundation of renewed efforts to eradicate this leading cause of childhood mortality (1, 2). The pharmacodynamic properties of clinically used ART derivatives [artesunate, artemether, and dihydroartemisinin (DHA)] can reduce the biomass of drug-sensitive parasites by four orders of magnitude every 48 hours (3), corresponding to a single cycle of asexual blood-stage *P. falciparum* development. The short half-life (typically <1 hour) of ART derivatives in plasma necessitates the use of longer-lasting partner drugs that can eliminate residual parasites once the ART component has dropped to subtherapeutic concentrations (4). The use of ACTs in expanded malaria control and elimination programs has yielded notable successes in recent years, contributing to an estimated 30%

reduction in global mortality rates in the past decade (5).

These impressive gains, however, are now threatened by the emergence of ART resistance, first detected in western Cambodia and now observed in Thailand, Vietnam, and Myanmar (6, 7). The severity of this situation is underscored by the fact that resistance to piperaquine, an ACT partner drug, is emerging in western Cambodia (8, 9). No alternative, fully effective first-line therapy is currently available to replace ACTs, should ART fail globally. Clinically, ART resistance is defined as a long parasite clearance half-life (the time it takes for the peripheral blood parasite density to decrease by 50%) after treatment with ART monotherapy or an ACT (6, 10, 11). This metric correlates with the percentage of early “ring-stage” parasites (0 to 3 hours after invasion of human erythrocytes) that survive a pharmacologically relevant exposure to DHA (the active metabolite

of all ARTs), as measured in the *in vitro* Ring-stage Survival Assay (RSA_{0-3h}) (12).

Recently, mutations in the propeller domain of the K13 gene were identified as candidate molecular markers of ART resistance (13). This gene resides on chromosome 13 of the *P. falciparum* genome, near regions earlier associated with slow parasite clearance rates (14–16). K13 belongs to the kelch superfamily of proteins, whose propeller domain harbors multiple protein-protein interaction sites and mediates diverse cellular functions, including ubiquitin-regulated protein degradation and oxidative stress responses (17). The K13 M476I mutation was first observed in Tanzanian F32 parasites that were exposed *in vitro* to escalating concentrations of ART over 5 years, yielding the F32-ART line (13, 18). [Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr. In the mutants, other amino acids were substituted at certain locations; for example, M476I indicates that methionine at position 476 was replaced by isoleucine.] Subsequent genomic analysis of Cambodian isolates identified four prevalent K13-propeller mutations (Y493H, R539T, I543T, and C580Y) that were associated with elevated RSA_{0-3h} survival rates *in vitro* and long parasite clearance half-lives (>5 hours) in patients (13, 19).

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Determining whether *K13*-propeller mutations confer ART resistance in clinical isolates and assessing the contributions of individual polymorphisms in distinct genetic backgrounds is essential to defining the underlying molecular mechanisms.

We developed zinc-finger nucleases (ZFNs) (20) to enable targeted genetic engineering of *K13* in newly culture-adapted Cambodian isolates and older established reference lines of *P. falciparum* (tables S1 and S2). ZFNs were introduced into cultured intra-erythrocytic parasites via electroporation with plasmids containing *K13* donor templates. ZFNs triggered double-stranded breaks in the *K13* genomic target locus of this haploid organism, leading to DNA resection and repair events that captured mutations delivered by pZFN^{*K13*}-hdhfr plasmids (fig. S1). Donor plasmids contained additional synonymous mutations that preclude ZFN binding while preserving the *K13*-translated amino acid sequence across that same stretch of DNA base pairs. These silent ZFN binding-site mutations protected the donor sequence and prevented the edited recombinant locus from being recleaved by the nucleases. Plasmids contained either the wild-type *K13* allele or one of several mutations (present in the six-blade *K13*-propeller domain) found in ART-resistant Cambodian isolates or F32-ART. This strategy successfully introduced or removed mutations in a set of *P. falciparum* clinical isolates from Cambodia, the epicenter of emerging ART resistance, as well as reference laboratory lines from distinct geographic origins (Fig. 1 and table S3). Of note, RSA_{0-3h} assays comparing parental and edited control parasites showed no difference if only the binding-site mutations were introduced into the *K13*-propeller domain, indicating that these synonymous mutations were phenotypically silent

(Fig. 1 and fig. S2). Independent assays with the same parasite lines tested by our different groups yielded consistent survival rates between laboratories (fig. S3).

Using donor plasmids containing a wild-type *K13*-propeller sequence and silent binding-site mutations, we generated a series of clones in which individual *K13* mutations were removed from ART-resistant Cambodian isolates. One of these isolates (Cam3.II) showed slow clearance after ART monotherapy (in vivo half-life 6.0 hours) (table S2). Parental Cam3.I^{R539T} and Cam3.II^{R539T} isolates harboring the R539T mutation showed 40 to 49% RSA_{0-3h} survival, whereas edited Cam3.I^{rev} and Cam3.II^{rev} clones carrying the reverted wild-type allele showed only 0.3 to 0.7% survival (Fig. 2, A and B, and table S4). These highly significant differences in the survival rates of ring-stage parasites exposed to elevated DHA concentrations confirm the importance of R539T in mediating in vitro ART resistance in Cambodian isolates. Significant reductions in RSA_{0-3h} survival rates were also observed upon removal of I543T (43% in Cam5^{I543T} versus 0.3% in Cam5^{rev}) (Fig. 2C) and C580Y (13% in Cam2^{C580Y} versus 2.4% in Cam2^{rev}) (Fig. 2D).

We also assessed the impact of introducing *K13* mutations into a fast-clearing Cambodian isolate (CamWT; in vivo half-life 3.7 hours) (table S2), the Cam3.II^{rev} clone, and three reference lines (VI/S, F32-TEM, and FCB). CamWT and Cam3.II^{rev} parasites harboring wild-type *K13* alleles showed 0.6 to 0.7% RSA_{0-3h} survival, whereas the corresponding C580Y-edited clones yielded 9 and 24% survival, respectively (Fig. 2, E and F). Introducing R539T into VI/S caused a similar increase in RSA_{0-3h} survival (0.3 to 21%) (Fig. 2G and table S4). Editing F32-TEM to express M476I

caused a moderate increase in RSA_{0-3h} survival (<0.2% in F32-TEM to 1.7% in F32-TEM^{M476I}) (Fig. 2H). We also observed modest in vitro resistance in FCB parasites edited to express C580Y, with RSA_{0-3h} survival increasing from 0.3% in the parental line to 1.9% in FCB^{C580Y} parasites (Fig. 2I). This result differs from a recent study of the use of Cas9 in *P. falciparum*, which reported a greater increase in RSA_{0-3h} survival (11 to 15%) in two clones engineered to express K13 C580Y (21). That report used the drug-sensitive NF54 strain—which was isolated decades before ART use and the emergence of resistance (22)—and did not examine additional mutations or assess the impact of removing *K13* mutations from ART-resistant clinical isolates.

In contrast to the substantial changes we observed in the RSA_{0-3h}, standard in vitro dose-response measurements by use of parental and *K13*-edited VI/S and Cam3.II parasites revealed no effect of R539T or C580Y on DHA or artesunate median inhibitory concentration (IC_{50}) values (fig. S4). This finding is consistent with earlier studies that showed no correlation between IC_{50} values and clinical ART resistance (6, 10, 12).

We subsequently investigated whether individual mutations confer different levels of ART resistance in the RSA_{0-3h}. In the Dd2 reference line, the introduction of M476I, R539T, or I543T mutations conferred considerably higher degrees of resistance than those of Y493H and C580Y (10 to 30% versus 2 to 4% survival, respectively) (Fig. 2J and table S4). These data corroborate the recent observation of higher levels of in vitro resistance in Cambodian isolates containing the R539T mutation as compared with Y493H or C580Y (23).

The relatively modest increase in survival of C580Y-expressing Dd2 parasites compared with

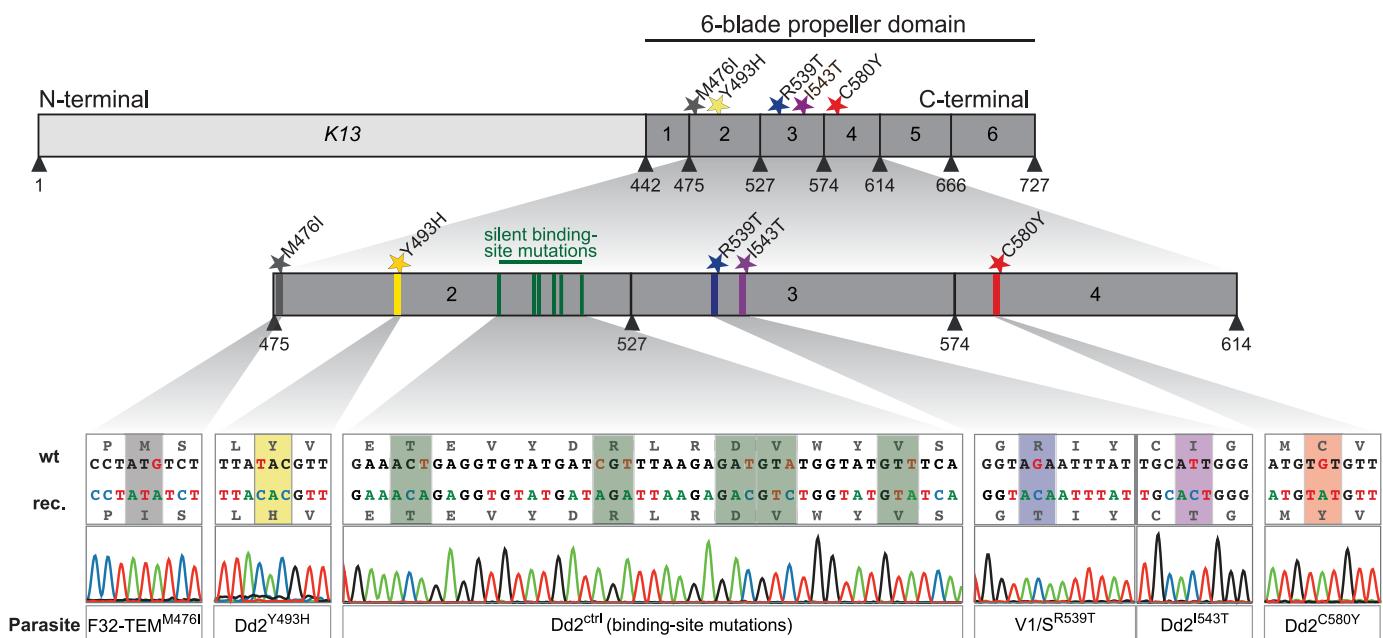


Fig. 1. Genetic modification of the *K13*-propeller domain. Location of *K13*-propeller mutations and sequencing results showing the insertion of individual mutations into recombinant parasites used in the RSA_{0-3h}. Dd2^{ctrl} parasites contain only synonymous, phenotypically silent binding-site mutations and showed 0.7% survival rates, which is equivalent to those of parental Dd2 parasites (fig. S2).

R539T- and I543T-expressing clinical isolates and edited clones was quite unexpected, given that C580Y has rapidly become the predominant mutant allele in western Cambodia (7, 13). We thus explored the impact of C580Y in different genetic backgrounds. Introducing C580Y conferred greater levels of resistance in three Cambodian isolates as compared with Dd2 and FCB parasites (Fig. 2K), suggesting a role for additional parasite factors in augmenting K13-mediated resistance in these contemporary field isolates. The disparity between relatively low in vitro resistance conferred by C580Y and its widespread dissemination in Cambodia might be explained by a lower fitness cost or increased transmission potential of C580Y-expressing parasites, or by the parasite genetic background.

Cambodian parasites are specifically characterized by sympatric subpopulations that show only limited genetic admixture and that generally harbor distinct K13 mutations (16). These findings suggest that K13 mutations might have arisen preferentially on backgrounds with favorable genetic factors. In this context, recent comprehensive analyses of K13 mutations across multiple sites in Southeast Asia have documented a series of additional mutations associated with slow clearance rates in Cambodia, Thailand, Myanmar, Laos, and Vietnam (7, 24). K13 mutations have also been observed in African isolates (7, 25, 26), although none of these correspond to the most prevalent mutations in Cambodia, and ART or ACT treatments in African sites continue to show a

high level of efficacy (7). A recent deep-sequencing study of the K13-propeller domain in more than 1110 *P. falciparum* infections collected from 14 sites across sub-Saharan Africa identified a large reservoir of naturally occurring K13-propeller variation, whose impact on artemisinin susceptibility is unknown and requires further investigation. These polymorphisms include one rare mutation previously observed in Cambodia (P553L) and several others (including A578S) close to known resistance-causing mutations in the propeller domain (26). Our gene-editing system can now be used to comprehensively dissect K13 polymorphisms across malaria-endemic regions and identify those that confer ring-stage ART resistance.

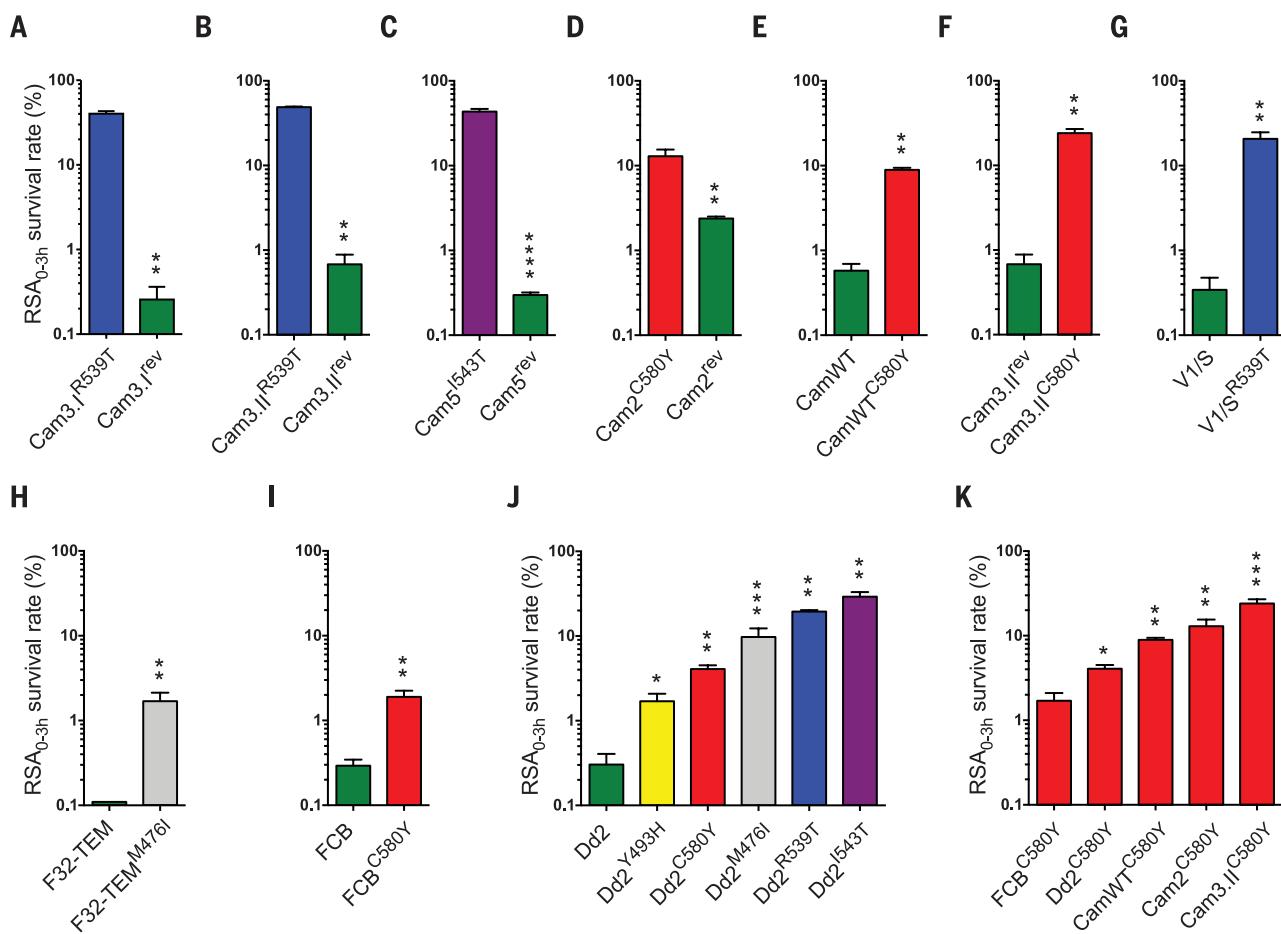


Fig. 2. K13-propeller mutations confer artemisinin resistance in clinical isolates and reference lines in vitro, as defined in the RSA_{0-3h}. Results show the percentage of early ring-stage parasites (0 to 3 hours after invasion of human erythrocytes) that survived a 6-hour pulse of 700 nM DHA (a pharmacologically relevant concentration of the active metabolite of ARTs), as measured by microscopy 66 hours later. Data show mean \pm SEM percent survival compared with control dimethyl sulfoxide-treated parasites processed in parallel. (A to D) RSA_{0-3h} survival for Cambodian isolates harboring native K13 mutations (shown in superscript) and ZFN-edited isogenic clones carrying wild-type K13 alleles (superscript "rev"). (E to I) RSA_{0-3h} survival for Cambodian isolates and reference lines harboring wild-type K13 alleles and ZFN-edited isogenic clones carrying individual K13 mutations (shown in superscripts). (J) Impact of different K13 mutations on RSA_{0-3h} survival in the Dd2 reference line, showing that I543T and R539T confer

the highest levels of resistance. (K) Introduction of C580Y into multiple Cambodian clinical isolates and reference lines, showing that this mutation confers varying degrees of in vitro resistance depending on the parasite genetic background. The geographic origins and known drug-resistance genotypes of these isolates and lines are provided in table S2. Results were obtained from 3 or 4 independent assays performed in duplicate (values provided in table S4; F32-TEM showed <0.2% RSA_{0-3h} survival). Two-sample *t* tests with unequal variances (performed with the STATA package) were used to assess for statistically significant differences between K13-edited clones and their comparator lines—the parental isolates listed on the left in (A) to (J) and the FCB^{C580Y} clone in (K) (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$). Statistical outputs (including calculations of the SE of the difference between the means of samples being compared and the *P* values) are listed in the supplementary materials.

Mode-of-action studies have shown that ARTs are active against all asexual blood stages of parasite development. In the more mature trophozoite stages, ARTs are activated after hemoglobin degradation and liberation of reactive heme whose iron moiety can cleave the endoperoxide linkage of these sesquiterpene lactone drugs (27). Activation generates free radicals that are thought to trigger oxidative stress and damage cellular macromolecules, including parasite membrane components, proteins, and neutral lipids (28, 29). Recent evidence suggests that hemoglobin degradation begins early after merozoite invasion, potentially providing a source of ART activator in ring-stage parasites (30). Our RSA_{0-3h} data support earlier evidence that reduced ring-stage susceptibility accounts for the clinical phenotype of slow parasite clearance after ART treatment (12, 31). K13 mutations might achieve this by protecting parasites from the lethal effects of ART-induced oxidative damage, potentially via a cellular pathway similar to antioxidant transcriptional responses regulated by the mammalian ortholog Keap1 (32). Our set of K13-modified isogenic parasites with different levels of ART resistance on distinct genetic backgrounds now enables a search for K13-interacting partners and delivers tools to interrogate the underlying mechanism.

Our data demonstrate a central, causal role for K13-propeller mutations in conferring ART resistance in vitro and provide a molecular explanation for slow parasite clearance rates in patients (6, 7, 10). By exposing greater parasite biomasses to ACTs in vivo, K13-propeller mutations may promote the evolution of partner drug resistance (8, 9) and higher-grade ART resistance. Our study thus offers a conclusive rationale for a global *K13* sequencing effort to track the spread of ART resistance and mitigate its impact on malaria treatment and control programs, particularly in hyperendemic regions in Africa.

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ACKNOWLEDGMENTS

D.A.F. gratefully acknowledges funding from the NIH (R01 AI09023). This study was supported in part by the Intramural Research Program of the National Institute of Allergy and Infectious Diseases, NIH, the French "Agence Nationale de la Recherche" (ANR-13-BSV3-0018-01 and the Laboratoire d'Excellence IBEID), and the Institut Pasteur, Division International (ACIP A-10-2010). Parental and transgenic parasite lines have been deposited and are being made available through BEI Resources (www.mrr.org) with the following accession numbers: MRA-1240, Cam3.IR539T (also known as IPC 5202); MRA-1252, Cam3.Irev; MRA-1241, Cam5I543T (also known as IPC 4912); MRA-1253, Cam5rev; MRA-1236, Cam2C580Y

(also known as IPC 3445); MRA-1254, Cam2rev; MRA-1250, CamWT; MRA-1251, CamWTC580Y; MRA-150, Dd2; MRA-1255, Dd2R539T. Parasite lines generated for this study will also be provided upon request from D.A.F. Requests for ZFNs should be directed to F.D.U. (Furnov@sangamo.com); a materials transfer agreement is required. We extend our gratitude to F. Ariey (Institut Pasteur, Paris) for his important contribution to initiating this study, I. McKeague and O. Lieberman (Columbia University Medical Center) for their statistical and scientific input, and E. Rebar and the Production Group at Sangamo BioSciences for ZFN assembly and validation. L.Z., S.L., P.D.G., and F.D.U. declare that they are full-time employees of Sangamo, which designed, validated, and provided the ZFNs used in this study. B.W., O.M.-P., F.B.-V., and D.M., are co-inventors on the pending patents US61/904651 and US62/062439, and N.K. is a co-inventor on the pending patent US62/062439. Both patents are filed by Institut Pasteur. These patents cover the use of K13 mutations as a molecular marker of *P. falciparum* ART resistance. Sangamo holds patents on engineered DNA-binding proteins and the use thereof in targeted genome engineering and gene-specific regulation. All other authors declare no competing financial interests.

SUPPLEMENTARY MATERIALS

www.sciencemag.org/content/347/6220/428/suppl/DC1
Materials and Methods
Figs. S1 to S4
Tables S1 to S5
Results of two-sample t tests with unequal variances
References (33–36)

5 September 2014; accepted 1 December 2014
Published online 11 December 2014;
10.1126/science.1260867

DRUG RESISTANCE

Population transcriptomics of human malaria parasites reveals the mechanism of artemisinin resistance

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Artemisinin resistance in *Plasmodium falciparum* threatens global efforts to control and eliminate malaria. Polymorphisms in the kelch domain–carrying protein K13 are associated with artemisinin resistance, but the underlying molecular mechanisms are unknown. We analyzed the *in vivo* transcriptomes of 1043 *P. falciparum* isolates from patients with acute malaria and found that artemisinin resistance is associated with increased expression of unfolded protein response (UPR) pathways involving the major PROSC and TRiC chaperone complexes. Artemisinin-resistant parasites also exhibit decelerated progression through the first part of the asexual intraerythrocytic development cycle. These findings suggest that artemisinin-resistant parasites remain in a state of decelerated development at the young ring stage, whereas their up-regulated UPR pathways mitigate protein damage caused by artemisinin. The expression profiles of UPR-related genes also associate with the geographical origin of parasite isolates, further suggesting their role in emerging artemisinin resistance in the Greater Mekong Subregion.

Artemisinin resistance in *Plasmodium falciparum* is spreading rapidly throughout Southeast Asia. Since it was first detected 7 years ago in Pailin, western Cambodia (1), artemisinin resistance has

become prevalent in other Cambodian provinces (2, 3), Thailand–Myanmar border areas (4, 5), and southern Vietnam (4, 6) and is emerging in southern Laos and central Myanmar (4). Artemisinin resistance threatens the

Chapitre III. Cartographie mondiale du polymorphisme du domaine en hélice du gène K13 de *P. falciparum*

1. Introduction

La récente identification de certaines mutations dans le domaine en hélice du gène *K13* comme déterminants majeurs de la résistance à l'ART chez *Plasmodium falciparum*⁸⁹ a offert la possibilité de surveiller l'étendue de cette résistance à l'échelle mondiale.

Cartographier la présence ou l'absence de parasites mutés à l'échelle planétaire est précisément l'objectif du projet KARMA (K13 Artemisinin Resistance Multicenter Assessment Consortium), un consortium international soutenu par l'Organisation Mondiale de la Santé (OMS) et réunissant 41 partenaires, dont 13 appartenant au Réseau International des Instituts Pasteurs, de 59 pays endémiques.

Nous avons analysé le polymorphisme de la séquence du domaine en hélice du gène *K13* de 14 037 échantillons de sang de patients infectés par *Plasmodium falciparum* (72% provenant d'Afrique, 19% d'Asie, 8% d'Amérique Latine et 1% d'Océanie). La majorité de ces échantillons (84.5%) proviennent de patients traités dans des sites sentinelles utilisés dans le cadre d'une surveillance nationale de la résistance aux antipaludiques. Tous ont été collectés après 2012 afin d'obtenir une vue d'ensemble de la situation la plus récente possible. Nous avons également étudié l'émergence et la dissémination des mutations par haplotypage des loci avoisinants le gène *K13*.

2. Résultats et discussion

Nous avons pu obtenir les séquences du gène *K13* de 13 157 échantillons (93.7%) et ainsi observer près de 180 mutations différentes, dont 70 nouvelles (103 connues), témoignant d'un polymorphisme très étendu du gène *K13*. La situation s'est avérée très différente selon

les continents, avec un grand nombre de mutations en Afrique mais avec des fréquences très faible (une mutation seulement, A578S, avec une fréquence >1%) et peu de mutations en Asie mais avec des fréquences beaucoup plus élevées.

Seules 9 mutations non-synonymes avaient une fréquence supérieure à 1% (C580Y, F446I, R539T, A578S, Y493H, P574L, P553L, N458Y, ET R561H) et 8 d'entre elles ont été associées à la résistance à l'ART. Par la méthode de recombinaison génétique décrite dans l'article précédent⁸⁹, nous avons inséré la mutation A578S dans la lignée de référence DD2 afin de vérifier *in vitro* si elle conférait de la résistance à l'ART. La souche obtenue, porteuse de la mutation A578S, s'est révélée sensible à l'ART en test RSA, suggérant donc que la résistance à l'ART n'a pas encore touché l'Afrique puisque cet allèle n'a pas non plus été associé au phénotype de résistance clinique.

Enfin, l'haplotypage des loci avoisinants a révélé l'existence de deux foyers distincts d'émergence en Asie du Sud-Est, l'un regroupant le Cambodge, le Vietnam et le Laos et l'autre le Myanmar, l'ouest de la Thaïlande et le sud de la Chine, chacun témoignant de multiples évènements d'émergence. Ces foyers d'émergence de parasites résistants à l'ART sont indépendants l'un de l'autre, ce qui tend à montrer que les stratégies internationales mises en place pour contenir la dissémination de la résistance ont été efficaces.

En définitive, notre article présente une méthodologie efficace pour l'amélioration de la surveillance de la résistance à l'ART, basée sur des tests moléculaires simples (génotypage du gène *K13* et haplotypage pour l'étude de la dissémination des mutants), des tests *in vitro*, et des études cliniques. Cette méthodologie pourrait également être étendue à d'autres marqueurs moléculaires, pour surveiller notamment la résistance aux drogues partenaires, afin de préserver leur efficacité en optimisant leur utilisation (rotation, trithérapie).

3. Article 3

A Worldwide Map of Plasmodium falciparum K13-Propeller Polymorphism.

D. Ménard, N. Khim, J. Beghain, A.A. Adegnika, M. Shafiu1-Alam, O. Amodu, G. Rahim-Awab, C. Barnadas, A. Berry, Y. Boum, M.D. Bustos, J. Cao, J.-H. Chen, L. Collet, L. Cui, G.-D. Thakur, A. Dieye, D. Djallé, M.A. Dorkenoo, C.E. Eboumbou-Moukoko, F.-E.-C.J. Espino, T. Fandeur, M.-F. Ferreira-da-Cruz, A.A. Fola, H.-P. Fuehrer, A.M. Hassan, S. Herrera, B. Hongvanthong, S. Houzé, M.L. Ibrahim, M. Jahirul-Karim, L. Jiang, S. Kano, W. Ali-Khan, M. Khanthavong, P.G. Kremsner, M. Lacerda, R. Leang, M. Leelawong, M. Li, K. Lin, J.-B. Mazarati, S. Ménard, I. Morlais, H. Muhindo-Mavoko, L. Musset, K. Na-Bangchang, M. Nambozi, K. Niaré, H. Noedl, J.-B. Ouédraogo, D.R. Pillai, B. Pradines, B. Quang-Phuc, M. Ramharter, M. Randrianarivelojosia, J. Sattabongkot, A. Sheikh-Omar, K.D. Silué, S.B. Sirima, C. Sutherland, D. Syafruddin, R. Tahar, L.-H. Tang, O.A. Touré, P. Tshibangu-wa-Tshibangu, I. Vigan-Womas, M. Warsame, L. Wini, S. Zakeri, S. Kim, R. Eam, L. Berne, C. Khean, S. Chy, M. Ken, K. Loch, L. Canier, V. Duru, E. Legrand, J.-C. Barale, B. Stokes, J. Straimer, B. Witkowski, D.A. Fidock, C. Rogier, P. Ringwald, F. Ariey, and O. Mercereau-Puijalon, for the KARMA Consortium*

New England Journal of Medicine. 2016 June 23

ORIGINAL ARTICLE

A Worldwide Map of *Plasmodium falciparum* K13-Propeller Polymorphisms

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ABSTRACT

BACKGROUND

Recent gains in reducing the global burden of malaria are threatened by the emergence of *Plasmodium falciparum* resistance to artemisinins. The discovery that mutations in portions of a *P. falciparum* gene encoding kelch (K13)-propeller domains are the major determinant of resistance has provided opportunities for monitoring such resistance on a global scale.

METHODS

We analyzed the K13-propeller sequence polymorphism in 14,037 samples collected in 59 countries in which malaria is endemic. Most of the samples (84.5%) were obtained from patients who were treated at sentinel sites used for nationwide surveillance of antimalarial resistance. We evaluated the emergence and dissemination of mutations by haplotyping neighboring loci.

RESULTS

We identified 108 nonsynonymous K13 mutations, which showed marked geographic disparity in their frequency and distribution. In Asia, 36.5% of the K13 mutations were distributed within two areas — one in Cambodia, Vietnam, and Laos and the other in western Thailand, Myanmar, and China — with no overlap. In Africa, we observed a broad array of rare nonsynonymous mutations that were not associated with delayed parasite clearance. The gene-edited Dd2 transgenic line with the A578S mutation, which expresses the most frequently observed African allele, was found to be susceptible to artemisinin in vitro on a ring-stage survival assay.

CONCLUSIONS

No evidence of artemisinin resistance was found outside Southeast Asia and China, where resistance-associated K13 mutations were confined. The common African A578S allele was not associated with clinical or in vitro resistance to artemisinin, and many African mutations appear to be neutral. (Funded by Institut Pasteur Paris and others.)

The authors' full names and academic degrees are listed in the Appendix. The authors' affiliations are listed in the Supplementary Appendix, available at NEJM.org. Address reprint requests to Dr. Ménard at the Institut Pasteur in Cambodia, 5 Monivong Blvd., P.O. Box 983, Phnom Penh, Cambodia, or at dmenard@pasteur-kh.org.

*A complete list of the investigators in the K13 Artemisinin Resistance Multicenter Assessment (KARMA) Consortium is provided in the Supplementary Appendix, available at NEJM.org.

N Engl J Med 2016;374:2453-64.

DOI: 10.1056/NEJMoa1513137

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INCREASED EFFORTS HAVE SUBSTANTIALLY reduced the global burden of malaria caused by *Plasmodium falciparum*,^{1,2} but the recent gains are threatened by emerging resistance to artemisinins, the cornerstone of current first-line combination treatment.^{1,3,4} Artemisinins are active against a large range of intraerythrocytic developmental stages, but their usefulness is curtailed by ring-stage resistance.^{5,6} Clinical artemisinin resistance, which was first documented in western Cambodia,^{7–10} is now prevalent across Southeast Asia and South China.^{11–17} Widespread artemisinin resistance would have dramatic consequences, since replacement therapies are limited and threatened by resistance.^{18–22}

Therapeutic efficacy studies are the standard method for determining the efficacy of antimalarial drugs,²³ but resource constraints restrict the numbers of sites and patients studied each year. The recent discovery of mutations in portions of a *P. falciparum* gene encoding kelch (K13)–propeller domains as the primary determinant of artemisinin resistance provided unprecedented opportunities for improving resistance monitoring.^{24,25} To date, 13 independent K13 mutations have been shown to be associated with clinical resistance,^{3,12,14,26–28} with evidence of independent emergence of the same mutation in different geographic areas.^{28,29} Four Asian mutations (C580Y, R539T, I543T, and Y493H) have been validated in vitro.^{24–27} In regions with no documented clinical artemisinin resistance, scattered studies have indicated that K13 mutations are rare.^{30–41} The few examples of reduced susceptibility to artemisinin-based combination therapy that have been reported in Africa were unrelated to K13 polymorphism, apart from three severe pediatric cases.^{42–44} No data are available for large regions of Africa, South America, Oceania, Central and East Asia, Indonesia, and the Philippines. The risk of the emergence or dissemination of resistance has spurred increased efforts to track artemisinin resistance in all geographic areas in which malaria is endemic. The use of a molecular indicator of artemisinin resistance is particularly timely, since rapid, large-scale screening is needed to provide an early warning about emergence or invasion events.^{45,46}

To assess the global distribution of K13 polymorphisms, we launched in 2014 the K13 Artemisinin Resistance Multicenter Assessment (KARMA) study, in which we analyzed parasites

that were collected from regions in which malaria is endemic, using a dedicated molecular toolbox and validation procedures for sequence data. Sequencing of the K13-propeller domain was combined with an analysis of flanking haplotypes to ascertain the origin and dissemination of specific mutations. We performed genome editing to confirm the phenotypic effect of the most frequent African A578S mutation. This worldwide survey was designed to provide critical information for drug policymakers and to outline methods for future surveillance activities.

METHODS

STUDY DESIGN, SAMPLING, AND OVERSIGHT

Investigators from Institut Pasteur in Paris and in Cambodia designed the study, which was coordinated by the Cambodian branch. According to the study design, the investigators planned to analyze 200 blood samples positive for *P. falciparum* that had been collected since 2012 and to contribute blood samples, K13 products obtained on polymerase-chain-reaction (PCR) assay, or K13 sequence data (Fig. S1 in the Supplementary Appendix, available with the full text of this article at NEJM.org). Sites that had few available samples or samples that were collected before 2012 were included if they were located in regions in which K13 diversity had not been documented. The study was approved by a national or institutional ethics committee or other appropriate authority at each site, and all investigators signed a declaration of ethical clearance.

Samples were obtained from patients seeking treatment at sites involved in national surveys of antimalarial drug resistance, from patients enrolled in therapeutic efficacy studies,²³ from asymptomatic participants who were enrolled in surveillance programs, and from travelers returning to Europe with malaria. Investigators at Institut Pasteur in Cambodia collected all biologic material and performed K13 sequence and haplotype analysis; they also conducted quality-control analyses, and they vouch for the accuracy and completeness of the molecular data. Investigators who conducted therapeutic efficacy studies vouch for the accuracy and completeness of the clinical data. The sponsors had no role in the study design or in the collection or analysis of the data. There was no confidentiality agreement between the sponsors and the investigators.

GENOTYPING

The Institut Pasteur in Cambodia provided reagents, blinded quality-control samples, and standard operating procedures as a K13 toolbox (Table S1 in the Supplementary Appendix). DNA extraction and amplification of the K13-propeller domain (codons 440–680, 720 bp)²⁴ were performed accordingly. PCR products were sequenced by Macrogen. We analyzed electropherograms on both strands, using PF3D7_1343700 as the reference sequence. We performed external quality assessment that included proficiency testing (in which 6 blinded quality-control samples were tested in each 96-well sequencing plate by each partner) and external quality control (in which 359 blood samples [2.6%] were independently retested) (Fig. S2 in the Supplementary Appendix). Isolates with mixed alleles were considered to be mutated for the purposes of mutation-frequency estimation.

The PF3D7_1337500 (K13_151) and PF3D7_1339700 (K13_159) loci were amplified (Table S2 in the Supplementary Appendix) and DNA sequences were analyzed as indicated above. Individual alleles were identified for each locus and haplotypes generated.

A578S GENE EDITING

We performed zinc-finger nuclease engineering, plasmid construction, and gene editing of the Dd2 line (which was collected in Indochina) with the A578S mutation.²⁵ We assessed the in vitro susceptibility to artemisinin of the Dd2 A578S mutation, the Dd2 parent, and the Dd2 C580Y mutation (as a positive control), using the ring-stage survival assay (RSA) performed in red cells that had been infected with the malaria parasite in its ring stage (the developmental stage that is associated with resistance to artemisinin) for an estimated 0 to 3 hours. The cells were then exposed to dihydroartemisinin (the main metabolite of all artemisinin derivatives), and drug susceptibility was measured at 72 hours.^{5,25}

GEOGRAPHICAL MAPPING

We calculated the proportion of parasites with a 3D7, wild-type allele (which is associated with artemisinin susceptibility) in each country and recorded the geospatial coordinates. To generate graphical maps, we interpolated data using ordinary kriging methods (or Gaussian process re-

gression) or inverse distance weighting. (Details about the methods are provided in the Supplementary Appendix.)

STATISTICAL ANALYSIS

We expressed quantitative data as medians and interquartile ranges or as means and 95% confidence intervals. We used the Mann–Whitney test or t-tests of independent samples to compare continuous variables and the chi-square test or Fisher's exact test to compare categorical variables. We obtained estimates of nucleotide diversity,⁴⁷ haplotype diversity,⁴⁸ and Tajima's D test⁴⁹ using DnaSP software, version 5.⁵⁰ All reported P values are two-sided, and a P value of less than 0.05 was considered to indicate statistical significance. Data were analyzed with the use of Microsoft Excel and MedCalc software, version 12.

RESULTS

SAMPLE COLLECTION

From May through December 2014, we gathered 14,037 samples from 163 sites in 59 countries. The samples included 11,854 (84.4%) from resident malaria patients in 40 countries, 1232 (8.8%) from residents with asymptomatic infections in 11 countries, and 951 (6.8%) from travelers returning with falciparum malaria from 40 countries (Fig. S3 in the Supplementary Appendix). The samples included those obtained from 2450 patients with malaria (2367 residents and 83 travelers) for whom follow-up data on day 3 were available (Table S3 in the Supplementary Appendix). We collected 4156 samples in 2012, 6440 samples in 2013, and 1671 samples in 2014 (>87% of all samples). Data regarding sample size, sex ratio, age, and parasitemia ranges according to study site are provided in Table S4 in the Supplementary Appendix.

K13-PROPELLER SEQUENCE POLYMORPHISMS

Sequences were generated for 13,157 (93.7%) samples; 880 samples from 35 sites had sequences that could not be interpreted because of poor quality or an insufficient quantity of DNA. Nearly all the samples (99.2%) contained a single K13 allele; 108 of 111 polyclonal infections were identified in samples obtained in Africa. The minor allele in a mixed sample was detected when its proportion was more than 20% (Fig. S4 in the Supplementary Appendix).

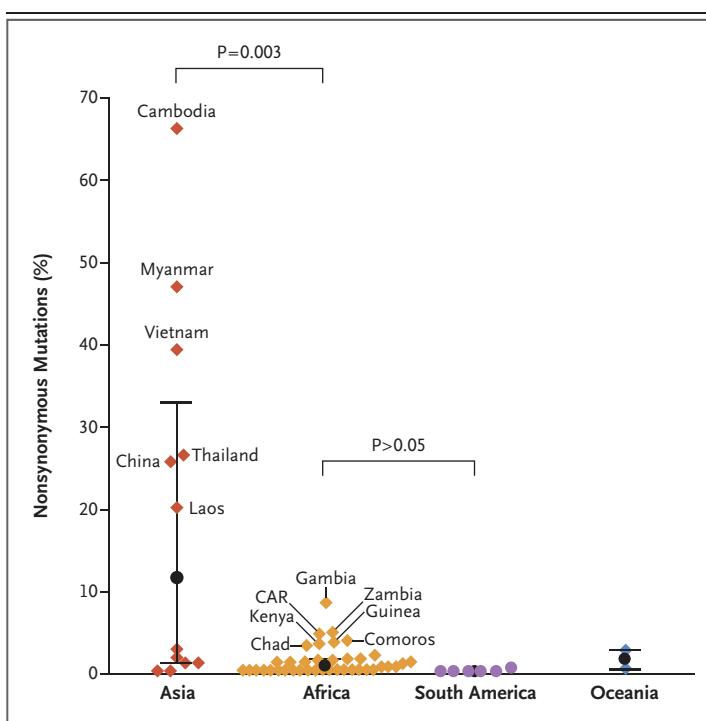


Figure 1. K13 Nonsynonymous Mutations, According to Country and Continent.

Shown are the percentages of nonsynonymous mutations that have been identified in the portion of the *Plasmodium falciparum* K13 gene encoding the kelch-propeller domain in Asia, Africa, South America, and Oceania. Synonymous mutations that do not modify the protein sequence are not indicated. At present, all the mutations that have been associated with resistance to artemisinin derivatives have resulted in nonsynonymous amino acid changes. The black circles indicate medians and the I bars interquartile ranges for each continent. K13 nonsynonymous mutations were not detected in 27 countries from which samples were obtained (19 in Africa, 2 in Asia, 1 in Oceania, and 5 in South America). Names are not shown (owing to a lack of space) for the following countries: in Asia: Afghanistan, Iran, Bangladesh, Nepal, Indonesia, and Philippines; in Africa: Cameroon, Congo, Democratic Republic of Congo, Equatorial Guinea, Gabon, Burundi, Ethiopia, Rwanda, Sudan, South Sudan, Somalia, Tanzania, Uganda, Madagascar, Angola, Malawi, Mozambique, South Africa, Zimbabwe, Benin, Burkina Faso, Ghana, Guinea Bissau, Ivory Coast, Liberia, Mali, Mauritania, Niger, Nigeria, Senegal, Sierra Leone, and Togo; in South America: Brazil, Colombia, Ecuador, French Guiana, Peru, and Venezuela; and in Oceania: Papua New Guinea and Solomon Islands. The percentages for Chad and Gambia were derived from a sample size of less than 50. Details regarding sampling and K13 diversity according to country are provided in Tables S4, S5, and S7 and Fig. S3 in the Supplementary Appendix. CAR denotes Central African Republic.

Overall, 1250 samples (9.5%) had 1295 K13 mutations, including 1097 (84.7%) with nonsynonymous mutations and 198 (15.3%) with synonymous mutations; 186 alleles were identified (Table S5 and Fig. S5 in the Supplementary Appendix).

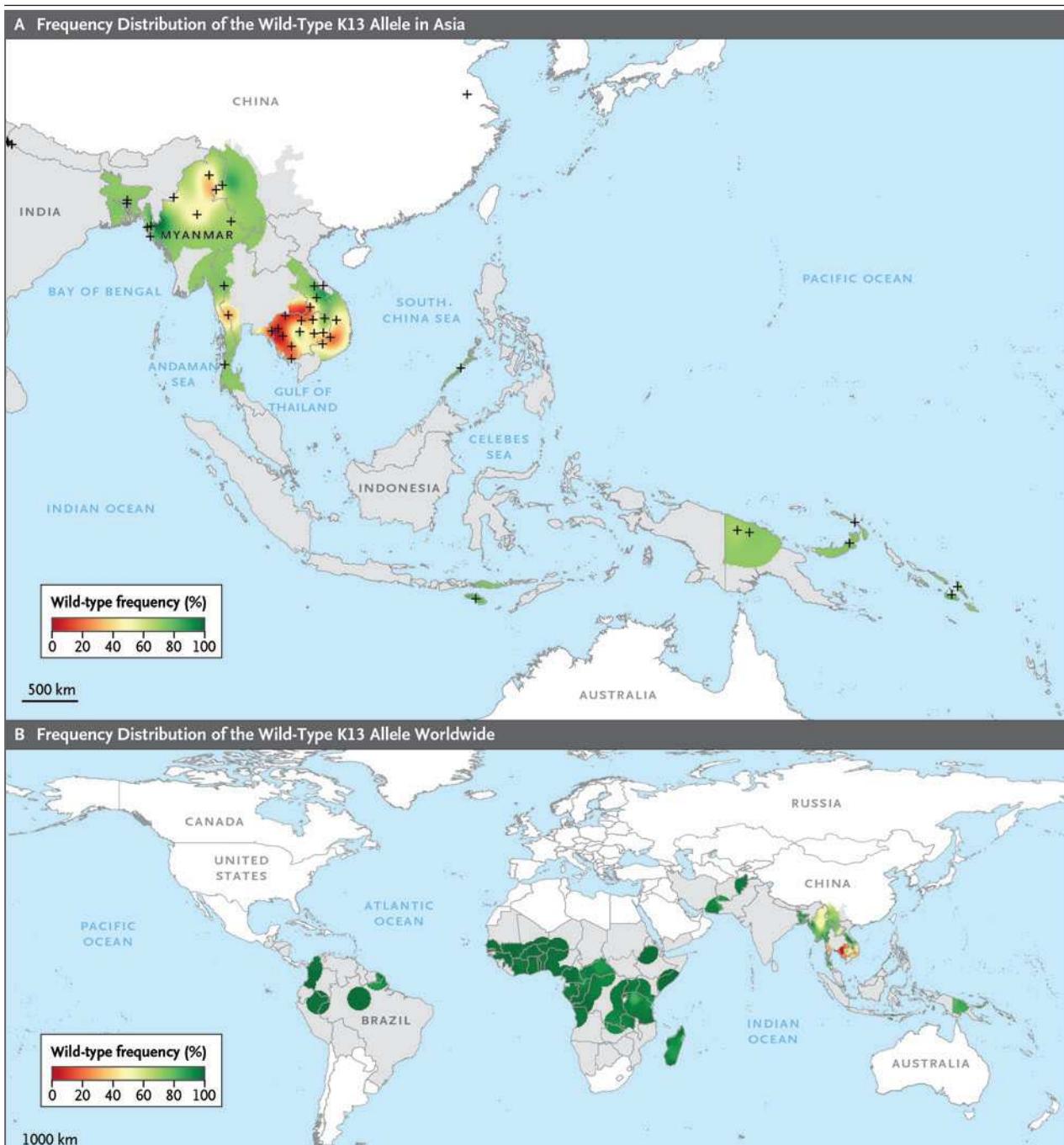
Among these alleles, there were 108 nonsynonymous mutations, of which 70 were newly identified and 38 were among 103 that had been reported previously.^{12,14,17,24,28,31-35,37-40,51-57} Only 9 nonsynonymous mutations (C580Y, F446I, R539T, A578S, Y493H, P574L, P553L, N458Y, and R561H) had a frequency of more than 1%; 72 alleles were observed only once (Tables S6 and S7 in the Supplementary Appendix).

There was marked geographic disparity in the proportion and distribution of K13 polymorphisms. We measured nucleotide diversity, haplotype diversity, and Tajima's D ratio to highlight the continent-specific frequency of mutant alleles (Fig. S6 in the Supplementary Appendix). Of the 1097 nonsynonymous mutations, 957 (87.2%) were observed in samples obtained in Asia, and 169 of the 198 synonymous mutations (85.4%) were observed in samples obtained in Africa.

CONTINENTAL DISTRIBUTION AND PROPORTION OF K13-PROPELLER MUTATIONS

The proportion of K13 nonsynonymous mutations was heterogeneous in Asia, ranging from fixed (>95%) to very high (80 to 94%) in western Cambodia (Fig. S7 in the Supplementary Appendix), to intermediate (40 to 50%) in Myanmar and Vietnam, to moderate (10 to 20%) in eastern Cambodia, Thailand, China, and Laos, to low (<5%) elsewhere (Fig. 1). In South America, Oceania, and Africa, K13 nonsynonymous mutations were uncommon, except for a few African countries (range, 3.0 to 8.3% in Gambia, Central African Republic, Zambia, Comoros, Guinea, Kenya, and Chad). K13 nonsynonymous mutations were not detected in 27 countries from which samples were obtained (19 in Africa, 2 in Asia, 1 in Oceania, and 5 in South America), as shown on maps of wild-type allele distribution (Fig. 2).

Individual K13 nonsynonymous mutations showed restricted geographic localization. In Asia, two distinct areas were identified, with different frequencies of individual mutations: one area that includes Cambodia, Vietnam, and Laos, where C580Y, R539T, Y493H, and I543T mutations were frequent or specific, and a second area that includes western Thailand, Myanmar, and China, where F446I, N458Y, P574L, and R561H mutations were specific (Fig. S8 in the Supplementary Appendix). The P553L allele was distributed in the two areas (Fig. 3).

**Figure 2. Frequency Distribution of the Wild-Type K13 Allele.**

Shown are the distributions of the wild-type K13 allele in Asia (Panel A) and around the world (Panel B). Areas in which malaria is endemic are shaded in gray, and areas that are considered to be malaria-free are shown in white. The mean frequency of the wild-type allele is indicated by the color code. In Panel A, the individual sites of sample collection are indicated with a cross. In Panel B, a 100-km radius was used for the area centered on each sampling site or on the capital city of the country if no specific site was used for sampling. Data regarding sampling methods and K13 diversity according to country are provided in Tables S4, S5, and S7 and Fig. S3 in the Supplementary Appendix.

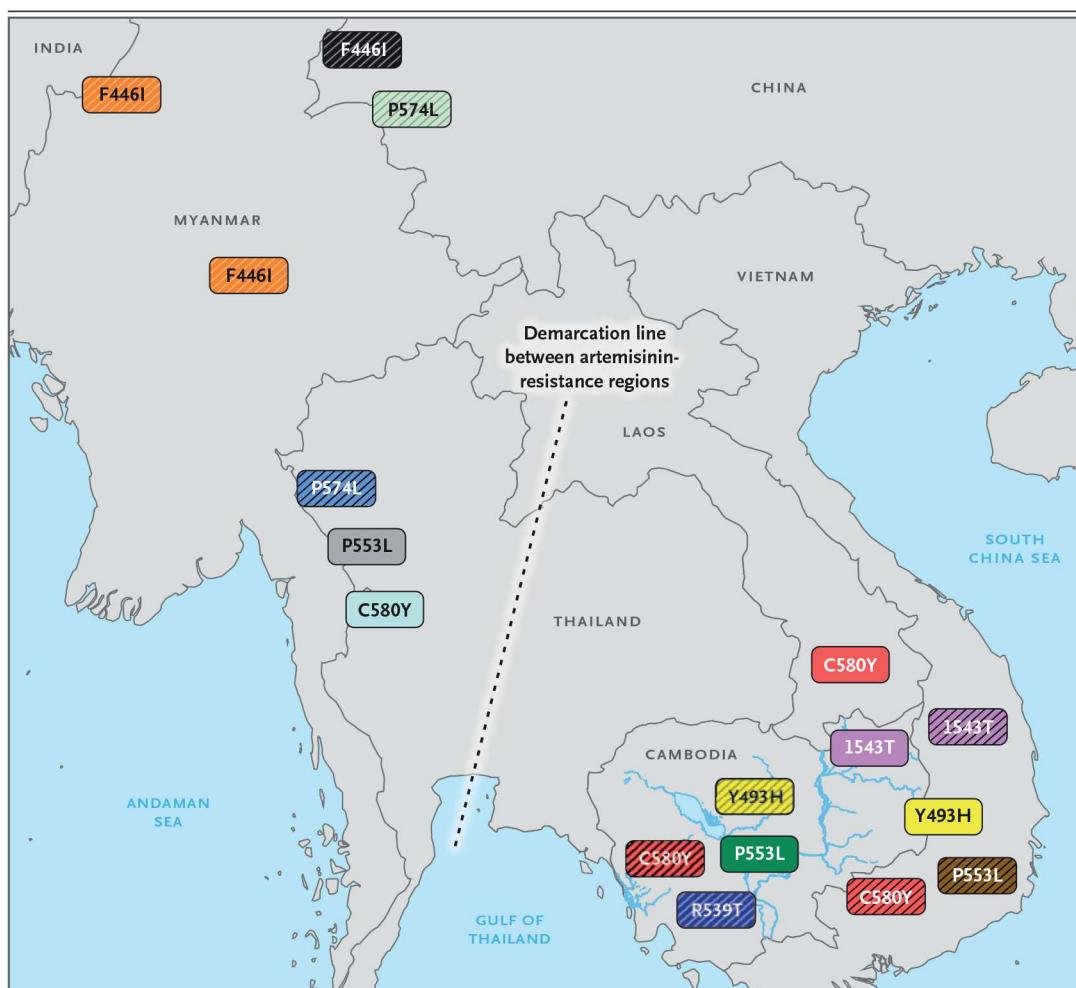


Figure 3. Overview of the Distribution of the Flanking Haplotypes of the C580Y, Y493H, R539T, I543T, P553L, P574L, and F446I Nonsynonymous Mutations in K13 in Two Regions in Asia.

Shown are two areas in Asia in which individual K13 nonsynonymous mutations (which are labeled in the colored boxes) were frequently identified: one area that includes Cambodia, Vietnam, and Laos and a second area that includes western Thailand, Myanmar, and China. The haplotype groups of C580Y, F446I, P574L, and P553L are shown in distinct colors. Similarly colored boxes indicate shared haplotypes, and hatched boxes indicate the presence of additional country-specific haplotypes. For example, C580Y haplotypes that are shared between Cambodia, Vietnam, and Laos (H29, H33, or H43) are shown in red, whereas the C580Y haplotype found in Thailand (H42) is shown in light blue. Full details about the distribution of haplotypes are provided in Table S9 and Fig. S9 in the Supplementary Appendix.

In Africa, no Asian artemisinin-resistance allele was observed among 9542 sequences, but 150 distinct alleles were identified, 92% of which were found in only one or two samples. Apart from A578S, V589I, S522C, V534A, F583L, and G665C, most alleles were Africa-specific and localized. A578S, which ranked fourth in abundance among mutant isolates, was observed in 1 sample from Thailand and 41 samples from Africa (Tables S6 and S7 in the Supplementary Appendix).

NEW EMERGENCE OR DISSEMINATION?

We investigated the genetic relatedness of isolates harboring the same frequent K13 mutation by assessing the polymorphism of two neighboring loci. We observed 10 alleles for K13_151 and 42 alleles for K13_159, which resulted in 80 flanking haplotypes (Tables S8 and S9 in the Supplementary Appendix). This assessment identified numerous emergence events alongside spreading of a small group of mutations for

artemisinin resistance (Fig. 3). Of 17 distinct C580Y haplotypes, 3 common haplotypes were distributed across Cambodia, Vietnam, and Laos and 14 in specific areas of Cambodia, Thailand, and Vietnam. Similar observations were made for the Y493H, R539T, and I543T haplotypes, with probable west-to-east dissemination for the Y493H and R539T mutations and movement from Vietnam to eastern Cambodia for I543T mutations. Three prevalent F446I haplotypes were distributed across Myanmar, and 8 haplotypes were localized within China or Myanmar. Site-specific localization was observed for the 5 P553L haplotypes, the 6 P574L haplotypes, and the 2 E605K and R561H haplotypes (Fig. S9 in the Supplementary Appendix). For the common African mutation A578S, we identified multiple independent events of emergence (31 different haplotypes among 35 isolates) (Fig. S10 in the Supplementary Appendix).

TESTING OF THE A578S ALLELE FOR ARTEMISININ RESISTANCE

We wanted to evaluate whether the A578S mutation had a selective advantage against artemisinin, since there have been reports about a conflicting phenotype of the allele,^{12,31,36,44} which has been found at multiple sites in Africa and Asia^{12,31,32,34-40,57} (Table S6 and Fig. S10 in the Supplementary Appendix). We therefore introduced the A578S mutation in the Dd2 line that had been used to investigate the effect of several Asian K13 mutations.²⁵ The Dd2 line with the A578S mutation was susceptible to artemisinin on RSA. The survival rate (mean [\pm SE], $0.29 \pm 0.11\%$) was equivalent to that in the parental line (mean, $0.14 \pm 0.03\%$), which was unlike the survival rate (mean, $4.01 \pm 0.16\%$) in the positive control Dd2 line with the C580Y mutation (Fig. S11 in the Supplementary Appendix).

K13 MUTATIONS AND POSITIVITY RATE

Our sampling included 2450 patients in whom the presence of parasites was assessed on day 3 after 7 days of artesunate monotherapy or a standard 3-day course of artemisinin-based combination therapy. Of these patients, 121 had positive parasite results on day 3 (Table S3 in the Supplementary Appendix). Of 40 nonsynonymous mutations that were detected in isolates obtained from these patients, only 8 were associated with positivity on day 3 (F446I, N458Y, N537D, R539T,

I543T, P553L, P574L, and C580Y); all these alleles were observed only in Southeast Asia and China (Fig. S12 in the Supplementary Appendix). No circulating blood-stage parasites were found on day 3 in samples obtained from 1533 African patients in 25 countries, except for 9 patients who were each carrying a wild-type allele. The 9 African patients who were carrying an A578S allele on day 0 were parasite-free on day 3.

DISCUSSION

In this study, we have attempted to profile the global distribution of K13 polymorphisms. The breadth of this survey, with 163 sites that were sampled for molecular mapping and 36 countries with data regarding therapeutic efficacy, led us to conclude that artemisinin resistance is confined to Southeast Asia and China, where resistance-associated K13 mutations have reached intermediate frequency to fixation. This status contrasts with the situation in South America, Oceania, the Philippines, and Central and South Asia, which are essentially free of nonsynonymous K13 mutations. We observed many highly diverse, low-frequency nonsynonymous mutations in Africa, although none of these mutations were associated with clinical artemisinin resistance, as assessed by the presence of parasites on day 3 after artesunate monotherapy or 3-day treatment with artemisinin-based combination therapy. We saw no evidence of invasion of Africa by Asian resistance-conferring alleles, a finding that was consistent with the results of previous smaller studies.^{35-38,40,41,44,58,59} Finally, no positive selection was observed aside from that in Asia, which suggests no immediate threat to artemisinin efficacy in most countries in which malaria is endemic.

In the two independent foci of resistance that have been identified in the region that includes Cambodia, Vietnam, and Laos and the one that includes western Thailand, Myanmar, and China, we observed many more examples of emergence of resistance-associated mutations than had been reported previously.^{28,29,55} This finding indicates that contemporary artemisinin resistance is a result of numerous independent emergence events involving the same mutations together with the dissemination of a small group of endemic mutations (Fig. 3). The endemic mutations are probably the oldest ones, as suggested by an analysis of our archived samples, which showed

that all four common contemporary C580Y haplotypes that were observed in Cambodia were already quite frequent 10 years earlier in Pailin province, the epicenter of the emergence of artemisinin-resistant parasites (Table S9 in the Supplementary Appendix).^{9,10} We observed no overlap between the sets of mutations and haplotypes in the two main resistance areas in Asia, which suggests the presence of different selective pressures in the two areas. Such differences may stem from the use of different artemisinin-based combination therapies as first-line treatment, different types of founder populations,^{29,59} or different epidemiologic ecosystems.

To date, 13 nonsynonymous mutations have been associated with slow parasite clearance,^{3,12,24,25,28,32} and 4 mutations have been validated as conferring an increased rate of ring-stage survival in drug-resistant field isolates in vitro²⁴ or in gene-edited parasite lines.⁴⁵ In the KARMA study, 8 nonsynonymous mutations that were observed in Southeast Asia and China — F446I, N458Y, N537D, R539T, I543T, P553L, P574L, and C580Y — were associated with positive results on day 3, findings that were consistent with data from previous studies that used parasite-clearance half-lives to identify artemisinin resistance^{12,14,24,26-28} and that provide further evidence of the positivity rate on day 3 as a sensitive indicator of clinical resistance to artemisinins.

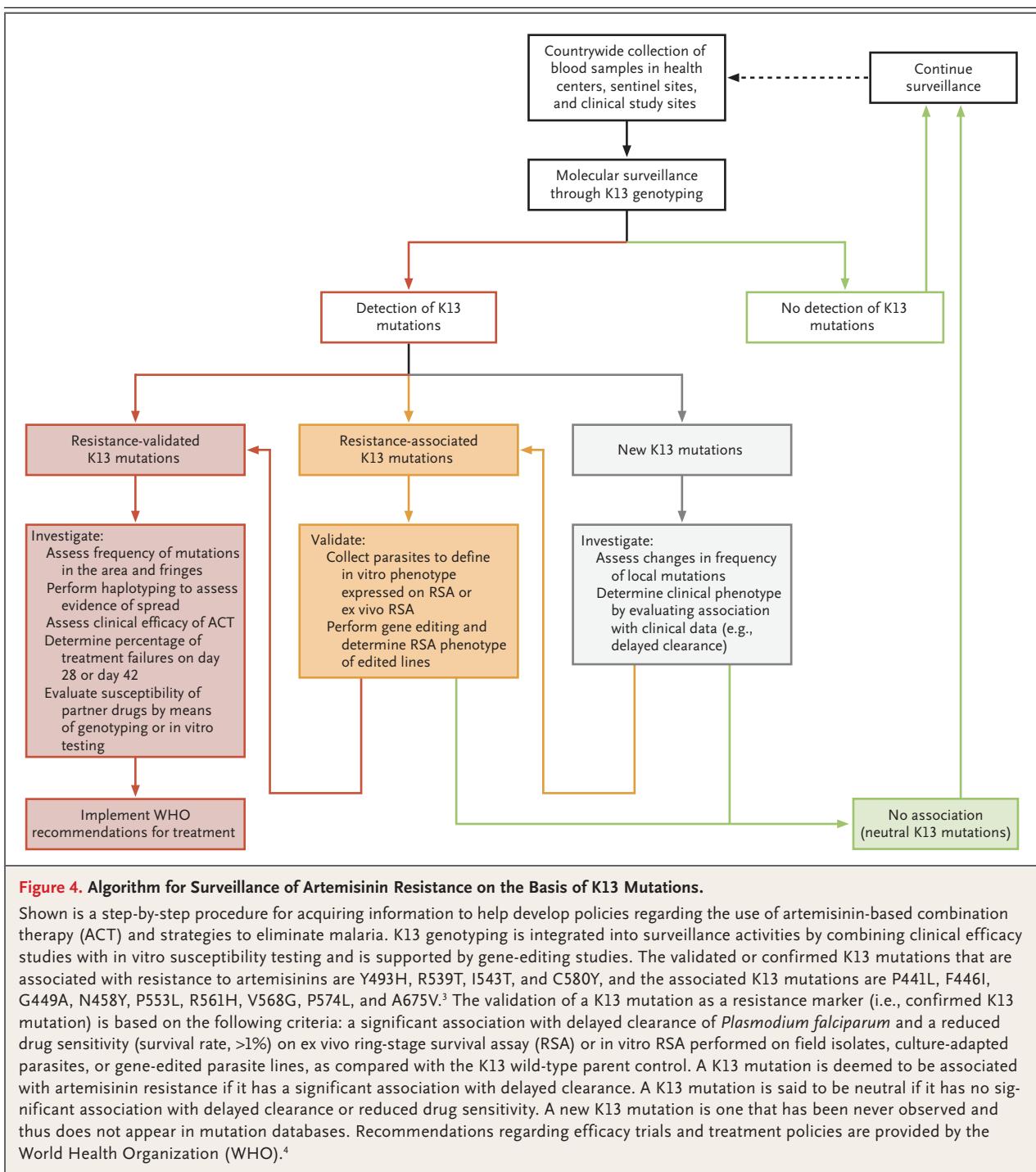
The A578S mutation that has been commonly observed in Africa^{12,35-40,44} and detected in India,³¹ Bangladesh,³⁴ and Thailand was sensitive on in vitro RSAs; this finding was unrelated to positivity on day 3. Numerous events of independent emergence were observed with no evidence of dissemination. These findings are consistent with the sensitive phenotype of one A578S Ugandan parasite as seen on RSA performed ex vivo (i.e., in samples obtained directly from patients)³⁶ or the lack of association with slow-clearing infections in earlier studies.^{12,31} Thus, except for one recent study involving a few severely ill children,⁴⁴ the available data suggest that A578S is not an artemisinin-resistance mutation.

Aside from nine patients who carried wild-type K13 alleles, there was no positivity on day 3 among African patients. This result is consistent with the finding that a large number of rare K13 alleles are neutral with respect to artemisinin susceptibility and with the lack of evidence of

artemisinin-driven selection on the K13 locus in Africa (Table S5 in the Supplementary Appendix). However, clinical detection of artemisinin resistance in Africa is complicated by the contribution of acquired immunity and maintained efficacy of the partner drugs (amodiaquine and lumefantrine) to parasite clearance.^{60,61} In vitro phenotyping with the use of RSA⁵ and allele-specific genome-editing studies²⁵ can provide important information about the potential phenotypic effect of nonsynonymous mutations with respect to artemisinin susceptibility, as we provided for A578S in this study. Similar approaches could be used to assess the potential effect of frequently observed K13 nonsynonymous mutations associated with clinical artemisinin resistance (e.g., F446I, N458Y, P574L, and P553L). Of 108 nonsynonymous mutations that were detected, we observed 72 only once, which was consistent with the results of earlier studies.^{35,37-41} The criteria for prioritizing further laboratory studies include the following: the frequent observation of a new allele with a nonsynonymous mutation, evidence of dissemination (since the absence of dissemination probably indicates that the mutation was lost to genetic drift or intrapopulation competition), and preliminary association with clinical data whenever possible.

Some clues about the phenotypic effect of newly discovered mutations might also be obtained from exploring specific metabolic changes (e.g., in phosphatidylinositol 3-phosphate levels⁶²) in parasites with K13 mutations or modeling the consequences of the mutations on K13 protein structure. The recently solved BTB-POZ (broad complex-tramtrack-bric-a-brac-poxvirus and zinc-finger) propeller structure of K13 protein domains (UniProtKB accession number, PDB AYY8) showed that F446, Y493, R539, and C580 are located in a beta sheet of their respective kelch domains (Fig. S13 in the Supplementary Appendix); these mutations should alter the overall structure (Fig. S14 in the Supplementary Appendix). We predict that A578S, located in the flexible junction between blade 3 and blade 4 of the beta sheet, has a limited effect on the propeller structure.

Our study has several weaknesses. First, the isolates that we evaluated represent only a convenience sample of those in the total population areas that we surveyed. Second, we lacked information about regions in which malaria is en-



demic such as India, where we needed to clarify the western boundary of resistance distribution. Third, in some countries, the numbers of available samples were small. The numbers of sites surveyed in East Africa, the Philippines, Indonesia, and South America need to be increased in

future studies, although we could still discern clear geographic patterns in the distribution of haplotypes. Investigators should be able to implement the K13 toolbox in the field. We expect that future surveillance will fill in the gaps of the existing map.

In conclusion, our study clarifies how K13 monitoring can assist future surveillance of artemisinin resistance (Fig. 4). In regions where resistance is established, K13 sequencing can map its geographic distribution and invasion of the fringes. The dissemination of K13 mutations can be conveniently assessed by determining the identity of flanking haplotypes, as we did here, which provides a more accessible approach than whole-genome sequencing²⁹ or microsatellite typing.⁵⁵ In resistance-free areas, molecular surveillance will need to detect possible invasion by known alleles associated with K13 resistance and track any temporal increase in the proportion and dissemination of newly identified nonsynonymous mutations. This strategy should identify foci to be targeted for further *in vitro* phenotyping and therapeutic efficacy studies as well as for surveillance of other candidate artemisinin-susceptibility loci.^{42,43} This effort can contribute information to characterize these areas with respect to potential artemisinin resistance and inform treatment guidelines before large-scale dissemination has occurred.

The views expressed in this article are those of the authors and do not necessarily reflect the official policies of the World Health Organization, the Department of the Navy, the Department of Defense, or the U.S. Government.

Supported by the Institut Pasteur Paris, Institut Pasteur International Division, Institut Pasteur Cambodia, and the World Health Organization; by a grant (ANR-10-LABX-62-IBEID) from the French Government Investissement d'Avenir program, Laboratoire d'Excellence "Integrative Biology of Emerging Infectious Diseases"; a grant from Natixis Banques; a grant (R01 AI109023, to Dr. Fidock) from the National Institutes of Health; grants from the Fiocruz Fundação Oswaldo Cruz, Fundação de Amparo à Pesquisa do Estado do Rio de Janeiro, Fundação de Amparo à Pesquisa do Estado do Amazonas, the Brazilian National Council for Scientific and Technological Development, the Agence Nationale de la Recherche (13-BSV3-0018-01 and 11-BSV7-009-01), the Austrian Federal Ministry of Science, Research, and Economy, the Calgary Laboratory Services, the Centre International de Recherches Médicales de Franceville, the European and Developing Countries Clinical Trials Partnership (CT-2004-31070-001),

the Drugs for Neglected Diseases Initiative, the Else Kroener Fresenius Stiftung, the Holger Poehlmann Stiftung, the European Community African-European Research Initiative "IDEA" (HEALTH-F3-2009-241642), the Fonds Wetenschappelijk Onderzoek, the Vlaamse Interuniversitaire Raad—Universitaire Ontwikkelingssamenwerking, the Belgian Technical Cooperation in Democratic Republic of Congo, the European Community Seventh Framework Program (FP7/2007-2013, 242095, and 223601), the European Commission (REGPOT-CT-2011-285837-STRONGER), the Ministère de la Santé Publique du Niger (Laboratoire National de Référence Résistance aux Antipaludiques), the Foundation of National Science and Technology Major Program (2012ZX10004-220), the French Ministry of Health (Institut National de Veille Sanitaire), the Global Fund to Fight AIDS, Tuberculosis and Malaria, the 5% Initiative program (French Ministry of Foreign Affairs, France Expertise Internationale, 12INI109), the Institut Pasteur de Madagascar, the Government of the Philippines, the Institut de Recherche pour le Développement, the Foundation des Treilles, the Délégation Générale pour l'Armement (PDH-2-NRBC-4-B1-402), the Institut Pasteur de Bangui, the International Society for Health Research and Training, the Malaria Research Initiative Bandarban, Vienna, International Centre for Diarrhoeal Disease Research, Bangladesh, the Médecins sans Frontières (Centre Opérationnel Paris, France), Medicines for Malaria Venture, the National Research Council of Thailand, the Thammasat University, the National Natural Science Foundation of China (81271870, 81361120405, and 81271863), the Natural Science Foundation of Jiangsu Province (BK20130114 and BK20150001), the Jiangsu Science and Technology Department (BM2015024), the National Institutes of Health (R01 AI11646601, AI109023, and ICEMR U19AI089702, U19AI089672), the Pasteur Institute of Iran, the Malaria Division of the Iranian Center for Diseases Management and Control, Public Health England (Malaria Reference Service Contract), the Government of Rwanda, the U.S. Department of Defense Armed Forces Health Surveillance Center, Global Emerging Infections Surveillance and Response System (P0463-14-N6), the Fogarty International Center of the National Institutes of Health training (D43 TW007393), the Mahidol-Oxford Research Unit, the Government of Japan (Science and Technology Agency, Agency for Medical Research and Development, Japan International Cooperation Agency, and Science and Technology Research Partnership for Sustainable Development), and the President's Malaria Initiative of the U.S. Agency for International Development.

Disclosure forms provided by the authors are available with the full text of this article at NEJM.org.

We thank all the patients, health workers, and research teams involved in the worldwide sample collection; Prof. C. Brechot and M. Jouan of Institut Pasteur for their support and advice; the shipping department team of the Institut Pasteur, Paris, for help in shipping the K13 toolbox; and Dr. Raymond Hui for providing information on the crystal structure of the K13 protein.

APPENDIX

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**Seconde Partie. Développement d'outils pour la détection et la surveillance
des résistances aux drogues partenaires : l'exemple de la piperaquine au
Cambodge**



Chapitre IV. Etat des lieux de la multi-résistance à l'ART et à la PPQ chez *P. falciparum* au Cambodge

1. Introduction

L'ouest du Cambodge est reconnu comme l'épicentre de la résistance multiple de *Plasmodium falciparum* aux antipaludiques⁹⁰. Les rapports les plus récents d'efficacité de l'association dihydroartémisinine-piperaquine (DHA-PPQ), la dernière combinaison à base d'artémisinine recommandée par l'OMS au Cambodge, ont incité à mener des investigations plus poussées. En effet, des études menées entre 2008 et 2010 ont montré une tendance à la baisse inquiétante des taux de guérison à l'ouest du Cambodge (de 89.4% à 75.0% à Pailin et de 98.7 à 89.3% à Pursat)⁹¹. Ces résultats suggèrent que des parasites résistants à la PPQ circulent dans cette région, bien qu'aucune preuve *in vitro* n'ait été apportée par les tests d'IC₅₀ réalisés (pas de différence significative entre les IC₅₀ à la PPQ d'isolats de patients guéris et ceux de patients en échec de traitement). Nous avons donc souhaité valider cette hypothèse et apporter des preuves cliniques et pharmacocinétiques robustes attestant de l'émergence de la résistance à la PPQ dans l'ouest du Cambodge.

Pour ce faire, nous avons évalué l'efficacité clinique de l'association DHA-PPQ à l'ouest et à l'est du Cambodge, sur 42 jours. Nous avons mesuré la concentration plasmatique de piperaquine à J7 et analysé la susceptibilité *in vitro* à la PPQ et à la MQ, le polymorphisme du gène *K13* et le nombre de copies du gène *Pfmdr1* des isolats à J0. Au total, 425 patients ont été recrutés entre 2011 et 2013.

2. Résultats et discussion

La proportion de patients avec des infections recrudescentes était significativement plus élevée à l'ouest qu'à l'est du Cambodge (15.4% contre 2.5% respectivement, $P < 10^{-3}$). Le

risque cumulatif d'échec de traitement à la DHA-PPQ était plus élevé pour les patients de l'ouest du Cambodge, avec une durée de survie moyenne de 40 jours (intervalle de confiance à 95% [95% CI], 39.1 à 40.9 jours), contre 41.8 jours pour les patients de l'est du Cambodge (95%CI, 41.7 à 42.0 jours) ($P < 10^{-6}$).

Les concentrations plasmatiques de PPQ à J7 se sont montrées indépendantes de l'issue thérapeutique. Par ailleurs, sur les 11 patients avec des infections recrudescentes dont la concentration plasmatique de PPQ à J7 a été mesurée, seuls 3 patients n'avaient pas une concentration adéquate (une concentration > 30 ng/mL étant considérée comme adéquate).

De la même manière, nous n'avons trouvé aucune différence entre la médiane des IC₅₀ à la PPQ des isolats provenant de patients guéris et celle des isolats de patients avec des infections recrudescentes. En revanche, la médiane des IC₅₀ à la MQ était significativement plus faible chez les isolats provenant de patients avec des infections recrudescentes (18.7 contre 39.7 nM; $P = 0.005$). Nous avons cependant été surpris de constater que seul 69% des tests *in vitro* nous ont fournis des courbes d'IC₅₀ interprétables pour la PPQ, contre 85% pour la MQ ($P = 0.03$). De plus, la proportion de courbes d'IC₅₀ non-interprétables était significativement plus élevée pour les isolats de patients avec des infections recrudescentes que pour les isolats de patients guéris, et ce uniquement pour la PPQ (77.8% contre 21.8% [$P < 10^{-8}$] pour la PPQ et 22.2% contre 12.1% pour la MQ), suggérant que ce format de test *in vitro* n'est pas adapté à l'étude de la susceptibilité à cette drogue. Il s'est également avéré que l'amplification du gène *Pfmdr1* avait un impact significatif sur les IC₅₀ à la fois à la PPQ et à la MQ : la médiane des IC₅₀ à la MQ était de 43.6 nM pour les isolats avec une seule copie de *Pfmdr1* contre 95.1 nM pour ceux avec un nombre de copies > 1 ($P < 10^{-3}$), et la médiane des IC₅₀ à la PPQ était de 48.0 nM pour les isolats avec une seule copie contre 40.0 nM pour ceux avec un nombre de copies > 1 ($P = 0.0004$).

Enfin, le facteur de risque le plus significativement associé aux échecs de traitement à la DHA-PPQ était la présence de mutation dans le gène *K13* (odds ratio [OR], 17.5; 95% CI, 1 à 308; $P = 0.04$).

Notre étude confirme que des parasites résistants à la PPQ circulent désormais à l'ouest du Cambodge, où la résistance à l'artémisinine est aujourd'hui fixée ($>85\%$ de parasites mutés dans le gène *K13*). Comme mesure immédiate, il a été décidé de remplacer la DHA-PPQ par l'AS-MQ dans les régions où la DHA-PPQ n'est plus efficace et où les parasites ne présentent pas (plus) d'amplification du gène *Pfmdr1*. Toutefois, il est possible que cette mesure ne soit efficace que provisoirement, et que des parasites résistants à la fois à la pipéraquine et à la méfloquine finissent par émerger. De plus, il est évident que l'absence à la fois d'un phénotype *in vitro* fiable, corrélé aux données *in vivo*, et d'un marqueur moléculaire pour la résistance à la PPQ constitue une lacune considérable rendant la surveillance de la résistance à la PPQ particulièrement compliquée. Il est désormais urgent de développer de nouveaux outils.

3. Article 4

Evidence of Plasmodium falciparum Malaria Multidrug Resistance to Artemisinin and Piperaquine in Western Cambodia: Dihydroartemisinin-Piperaquine Open-Label Multicenter Clinical Assessment.

Leang R, Taylor WR, Bouth DM, Song L, Tarning J, Char MC, Kim S, Witkowski B, Duru V, Domergue A, Khim N, Ringwald P, Menard D.

Antimicrob Agents Chemother. 2015 Aug;59(8):4719-26. doi: 10.1128/AAC.00835-15. Epub 2015 May 26.

Evidence of *Plasmodium falciparum* Malaria Multidrug Resistance to Artemisinin and Piperaquine in Western Cambodia: Dihydroartemisinin-Piperaquine Open-Label Multicenter Clinical Assessment

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Western Cambodia is recognized as the epicenter of *Plasmodium falciparum* multidrug resistance. Recent reports of the efficacy of dihydroartemisinin (DHA)-piperaquine (PP), the latest of the artemisinin-based combination therapies (ACTs) recommended by the WHO, have prompted further investigations. The clinical efficacy of dihydroartemisinin-piperaquine in uncomplicated falciparum malaria was assessed in western and eastern Cambodia over 42 days. Day 7 plasma piperaquine concentrations were measured and day 0 isolates tested for *in vitro* susceptibilities to piperaquine and mefloquine, polymorphisms in the *K13* gene, and the copy number of the *Pfmdr-1* gene. A total of 425 patients were recruited in 2011 to 2013. The proportion of patients with recrudescent infections was significantly higher in western (15.4%) than in eastern (2.5%) Cambodia ($P < 10^{-3}$). Day 7 plasma PP concentrations and median 50% inhibitory concentrations (IC_{50}) of PP were independent of treatment outcomes, in contrast to median mefloquine IC_{50} , which were found to be lower for isolates from patients with recrudescent infections (18.7 versus 39.7 nM; $P = 0.005$). The most significant risk factor associated with DHA-PP treatment failure was infection by parasites carrying the *K13* mutant allele (odds ratio [OR], 17.5; 95% confidence interval [CI], 1 to 308; $P = 0.04$). Our data show evidence of *P. falciparum* resistance to PP in western Cambodia, an area of widespread artemisinin resistance. New therapeutic strategies, such as the use of triple ACTs, are urgently needed and must be tested. (This study has been registered at the Australian New Zealand Clinical Trials Registry under registration no. ACTRN12614000344695.)

For decades, western Cambodia has been the focus of multidrug-resistant *Plasmodium falciparum* malaria and has seen the demise of chloroquine (1960s), sulfadoxine-pyrimethamine (1970s), and mefloquine (1990s) treatments (1–4). The combination of nonfixed artesunate plus mefloquine (AS-MQ) was the first artemisinin-based combination therapy (ACT) to be introduced in Cambodia, in 2000. However, when tested as early as 2001 in the western Cambodian provinces of Pailin and Battambang, AS-MQ and artemether-lumefantrine (AL) had efficacy rates below 90% (5–7). In 2008, artemisinin-resistant *P. falciparum* was observed in clinical studies, first in Battambang (8) and later in the adjoining provinces of Pailin (9) and Pursat (10). Retrospective molecular epidemiology investigations, looking for mutations in the propeller domain of the *K13* gene as the molecular marker of artemisinin resistance, confirmed that artemisinin-resistant parasites were already highly prevalent (>60%) in Pailin province in 2001 (11).

Piperaquine (PP), a 4-aminoquinoline bisquinoline with a half-life of ~9 days, was originally developed in France and is active against the erythrocytic stage of malaria parasites. In the 1970s to 1980s, PP alone was used as prophylaxis and treatment in the areas of malaria endemicity in southern China, where, according to some reports, observed rates of *P. falciparum* resistance were frequently ≥60% (12–14). Since 2008, dihydroartemisinin plus piperaquine (DHA-PP), the latest ACT to be recommended by the WHO, has been used as the first-line treatment for uncomplicated falciparum malaria in the western Cambodian provinces;

in 2010, it was extended to other provinces. Several studies have demonstrated that this ACT was safe and highly efficacious against falciparum malaria in Cambodia (15, 16), in Asia (17–20), and elsewhere (21). However, reports from DHA-PP therapeutic efficacy studies (42-day follow-up) performed from 2008 to 2010 have shown worrying downward trends in cure rates, from 89.4% to 75.0% in Pailin and from 98.7% to 89.3% in Pursat in western Cambodia, while cure rates in the provinces of Preah Vihear (northern Cambodia) (100% in 2009) and Ratanakiri (northeast

Received 8 April 2015 Returned for modification 14 May 2015

Accepted 20 May 2015

Accepted manuscript posted online 26 May 2015

Citation Leang R, Taylor WRJ, Bouth DM, Song L, Tarning J, Char MC, Kim S, Witkowski B, Duru V, Domergue A, Khim N, Ringwald P, Menard D. 2015. Evidence of *Plasmodium falciparum* malaria multidrug resistance to artemisinin and piperaquine in western Cambodia: dihydroartemisinin-piperaquine open-label multicenter clinical assessment. *Antimicrob Agents Chemother* 59:4719–4726. doi:10.1128/AAC.00835-15.

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Supplemental material for this article may be found at <http://dx.doi.org/10.1128/AAC.00835-15>.

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doi:10.1128/AAC.00835-15

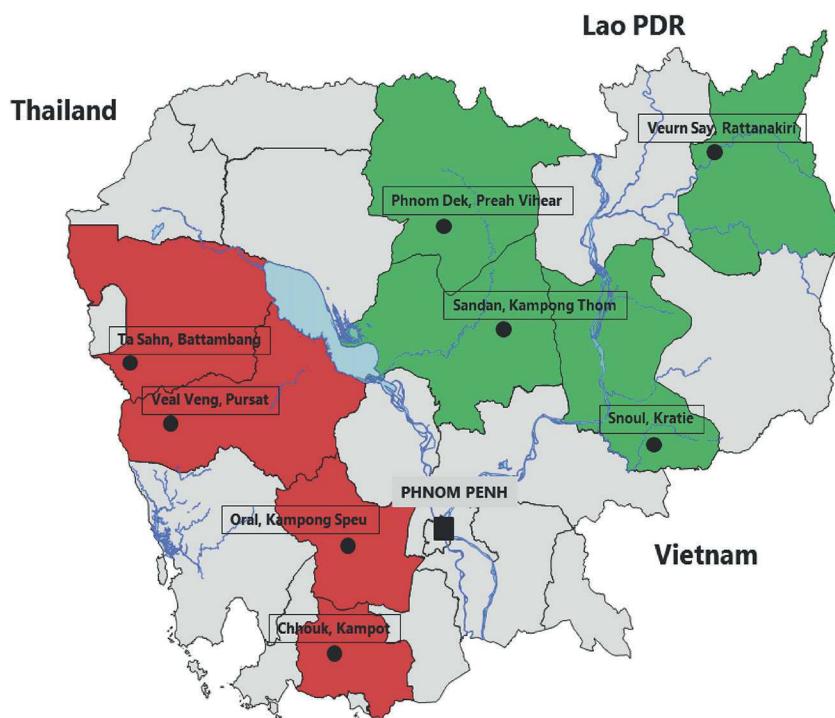


FIG 1 Map of Cambodia showing study sites and other provinces where DHA-PP has been tested. Western and eastern provinces are shown in red and green, respectively. Black dots mark the study sites (city and province names).

Cambodia) (100% in 2009 to 2010) remained high (22). This suggests that parasites resistant to PP are circulating in western Cambodia, although no evidence of *in vitro* resistance to PP has been observed. The median IC₅₀ (50% inhibitory concentrations, i.e., drug concentrations that inhibit parasite growth by 50%) of PP did not differ significantly between cured patients and patients with recrudescent infections in western provinces or between western and eastern provinces (22). These findings were later confirmed by Lim et al., who showed similar median PP IC₅₀ in Pursat, Preah Vihear, and Ratanakiri provinces (23). More recently, data from a small number of patients enrolled in Oddar Meanchey province (northern Cambodia) confirmed declining DHA-PP cure rates, to 64% and 53% (per-protocol efficacy) (24, 25); the PP IC₅₀ were similar for day 0 isolates and isolates collected on the day of recrudescence (25). Concentrations of PP in blood on day 7, to confirm adequate drug absorption, were not measured, and parasites were not assessed for evidence of artemisinin resistance using the *in vitro* ring-stage survival assay (11).

To date, validated *in vitro* phenotypic tests and reliable molecular markers of PP resistance are lacking, and there is only limited evidence for the emergence of PP resistance from *in vivo* tests. Here we report robust clinical and pharmacokinetic evidence of PP resistance and high rates of artemisinin resistance in western Cambodia.

MATERIALS AND METHODS

Study sites and patients. Our study was conducted at health centers at eight sites over 3 years (2011 to 2013) in western (Battambang, Pursat, Kampot, and Kampong Speu) and eastern (Kratie, Preah Vihear, Ratanakiri, and Kampong Thom) Cambodian provinces (Fig. 1). We did a prospective multicenter open-label study with dihydroartemisinin-piper-

aquine (DHA-PP) (Duo-Cotexin; 40 mg DHA and 320 mg PP; Zhejiang Holley Nanhu Pharmaceutical Co. Ltd., Jiaxing, Zhejiang province, China) for the treatment of acute uncomplicated symptomatic *P. falciparum* malaria in children and adults. The study protocol was adapted from the 2009 WHO protocol for assessment of the efficacy of antimalarial treatment (26). Patients were followed up for 42 days. The primary objective was to assess the cumulative risk of recrudescence of *P. falciparum* at day 42, after PCR correction, globally and between patients from western and eastern Cambodian sites. The secondary objective was to evaluate the proportions of parasitemic patients on days 1, 2, and 3. PP concentrations in blood at day 7, a proxy of adequate PP exposure, were measured in a subset of randomly selected patients with recrudescent infections and cured patients (see the supplemental material) (27). Risk factors associated with *P. falciparum* recrudescence after DHA-PP treatment were also evaluated.

The studies were approved by the Ethics Review Boards of the National Ethics Committee at the National Institute of Public Health, Phnom Penh, Cambodia, and the Ethics Review Committee of the WHO Regional Office for the Western Pacific. Written informed consent was obtained from adult patients and parents or guardians of enrolled children. The trial was registered at the Australian New Zealand Clinical Trials Registry (registration no. ACTRN12614000344695).

Patients. Patients aged >2 years with slide-confirmed falciparum malaria mono-infection (500 to 200,000 asexual parasites/ μ l) and fever (axillary temperature, $\geq 37.5^\circ\text{C}$) or a history of fever in the previous 24 h who presented to the study site were eligible for enrollment. Pregnant or lactating women and females 12 to 18 years old were excluded, as were patients with one or more signs of severe or complicated malaria (according to the WHO definition), severe malnutrition, concomitant febrile illness, significant underlying disease, hypersensitivity, or contraindication to DHA-PP.

Procedures. After enrollment, medical histories were recorded and physical and malaria blood film examinations performed. Blood from a

finger prick was collected for thick/thin blood films and parasite genotyping along with 5 ml venous blood (ACD [acid citrate dextrose] tube) for *in vitro* drug sensitivity testing. Falciparum malaria was diagnosed by microscopic examination of thick/thin blood films stained with Giemsa stain. Parasite counts were recorded as the number of parasites per 200 white blood cells, assuming a total white blood cell count of 8,000/ μ l. Two qualified microscopists read the slides, and the final parasite density was recorded as the mean of the two counts. A third reading was conducted if the parasitemia difference exceeded 50% or if there was positive-negative discordance.

Supervised DHA-PP was administered once daily for 3 days (day 0, 24 h, 48 h) by the research team. Dosing was based on body weight, in accordance with the national treatment guidelines, as follows: (i) for patients weighing <19 kg, 1 tablet/day; (ii) for those weighing 19 to 29 kg, 1.5 tablets/day; (iii) for those weighing 30 to 39 kg, 2 tablets/day; (iv) for those weighing >40 kg, 3 tablets/day. For children unable to swallow tablets, DHA-PP was dissolved in 5 ml of water. Patients were observed for 1 h postdosing and were redosed with a full or half dose if vomiting occurred within 30 min or between 31 and 60 min, respectively. Those who vomited after the second dose were withdrawn from the study and were given parenteral rescue treatment (intramuscular artemether). Patients with axillary temperatures of \geq 37.5°C were treated with paracetamol. Patients were seen daily to day 3 and then weekly for 6 weeks (day 42) for clinical examinations (axillary temperature, symptom check) and malaria blood films. Home visits were conducted if patients failed to come back for their follow-up appointments.

Patients failing DHA-PP therapy with recurrence of *P. falciparum* were retreated with artemether plus mefloquine as per national guidelines in district hospitals. For these patients, filter paper blood spots collected on day 0 and on the day of recurrent parasitemia were used to compare polymorphisms within the genes that encode MSP-1, MSP-2 (merozoite-specific proteins), and GLURP (glutamate-rich protein), as described previously, and these cases were recorded as reinfections or recrudescent infections (28).

PP concentrations were determined from 100- μ l blood spots (Whatman grade 31ET Chr cellulose chromatography paper) collected at day 7 from a finger prick, as described previously (29), for a subset of randomly selected patients with recrudescent infections and cured patients (4 per 10 samples from patients with recrudescent infections and 2 per 10 samples from cured patients). Briefly, blood samples were cut, and solid-phase extraction (SPE) was performed, followed by quantification by liquid chromatography (LC) and tandem mass spectrometry (MS-MS) (multiple reaction monitoring [MRM] mode) detection on an AB Sciex API 5000 triple quadrupole mass spectrometer. D6-PP was used as the internal standard. MRM transitions were m/z 535.10 to 288.15 (collision energy, 45 V) and m/z 541.00 to 294.14 (collision energy, 46 V) for PP and D6-PP, respectively. A new calibration curve was processed and analyzed with each batch of samples. The limit of detection was 1.0 ng/ml (signal-to-noise ratio, \geq 3:1), and the lower limit of quantitation was 3 ng/ml for PP (intra-assay precision, <20%; signal-to-noise ratio, \geq 10:1). The coefficients of variation during PP quantification ($n = 45$ determinations for each quality control concentration) were 7.57%, 3.98%, and 3.96% at 9 ng/ml, 40 ng/ml, and 800 ng/ml, respectively.

The *in vitro* susceptibilities of day 0 isolates to PP and mefloquine were assessed using the classical isotopic 48-h assay (22). Results were determined by nonlinear regression using ICEstimator software (<http://www.antimalarial-icestimator.net/>) and were expressed as 50% inhibitory concentrations (IC_{50}), defined as the concentrations at which 50% of the incorporation of [3 H]hypoxanthine was inhibited relative to that in drug-free control wells. In addition, after the extraction of DNA from day 0 blood samples (QIAamp DNA blood minikit; Qiagen, Valencia, CA), mutations in the *K13* propeller domain gene (*PF3D7_1343700*), recently associated with artemisinin resistance (11), and the *Pfmdr-1* copy number (22) were assessed.

Statistical analysis. Data were collected on a standard case record form, double entered on the WHO Microsoft Office Excel spreadsheet, and checked for concordance before being analyzed using Stata, version 12 (Stata Corporation, College Station, TX, USA). The Mann-Whitney U test was used for nonparametric comparisons. For categorical variables, proportions were examined by a chi-square or Fisher exact test. Odds ratios (ORs) were estimated using the Mantel-Haenszel test, and risk factors for DHA-PP treatment failure were assessed by a Cox-Mantel (log rank) test. The correlation between two continuous variables was assessed by the Spearman rho test. Two-sided P values of <0.05 were considered statistically significant.

The cumulative risk of failure at day 42 was assessed by survival analysis by the Kaplan-Meier method on an intention-to-treat basis. Patients lost during follow-up were censored on the last day of follow-up and were not regarded as treatment failures (TF). Patients failing to complete a 42-day follow-up due to recurrent *P. falciparum* parasitemia were regarded as treatment failures. The day 42 therapeutic responses after PCR correction were defined as either early treatment failure (ETF), late clinical failure (LCF), late parasitological failure (LPF), or adequate clinical and parasitological response (ACPR) (26). ETF, LCF, and LPF were defined as treatment failures. Treatment outcomes for patients from western and eastern Cambodia were compared by use of the Mantel-Haenszel log rank test, and the hazard ratio (HR) was calculated. The parasite reduction ratios (PRR) at 24 and 48 h were defined as 1 - (parasite count at 24 or 48 h/parasitemia at the onset of drug treatment) and expressed as a percentage. The day 3 positivity rate was defined as the proportion of patients who were malaria slide positive on day 3. A plasma PP concentration of \geq 30 ng/ml at day 7 was considered evidence of adequate PP exposure (27). The relationship between the concentration of PP in capillary blood spotted onto filter paper (cb) and that in plasma (pl) was calculated using the following correlation: $\log(e) \text{ PP}_{\text{pl}} = [\log(e) (\text{PP}_{\text{cb}} \times 0.974)] - 1.072$ (30).

RESULTS

From 2011 to 2013, a total of 425 patients with uncomplicated falciparum malaria were enrolled and were treated with DHA-PP at the eight study sites (147 patients in western Cambodia and 278 in eastern Cambodia). Among them, day 7 plasma PP concentrations were measured in 93 randomly selected samples from the group of patients with recrudescent infections (4 out of every 10 samples were selected [11 of 29 samples]) and the cured patient group (2 out of every 10 samples were selected [82 of 390 samples]) (62 patients in western Cambodia and 31 in eastern Cambodia) (see Fig. S1 in the supplemental material).

Differences in weight ($P < 10^{-4}$), age ($P = 0.002$), and DHA and PP doses (in milligrams per kilogram of body weight per day) ($P = 0.0004$ and 0.0003 , respectively) were observed between patients from western and eastern sites at day 0. The target doses of DHA (≥ 2 mg/kg/day) and PP (≥ 16 mg/kg/day) were achieved in 363/425 (85.4%) patients and were similar for western and eastern sites, as were the median plasma PP concentrations assessed for randomly selected patients at day 7 (Table 1). Only 62/425 (14.6%) patients received a PP dose below the recommended minimum of 16 mg/kg/day (range, 10.3 to 32.0 mg/kg/day), but no correlation between the day 7 plasma PP concentration and the dose of PP administered (in milligrams per kilogram) was found ($r = 0.04$; $P = 0.68$ [see Fig. S2 in the supplemental material]).

Among the patients enrolled, 419/425 completed the follow-up with an assigned clinical outcome. Six patients were lost to follow-up at days 3, 7, 14, 21 (two patients), and 35 (four from western and two from eastern Cambodia). Over the 42-day follow-up, parasitemia recurred in 6.9% (29/419) of patients (95% confidence interval [95% CI], 4.6% to 9.9%). The proportion of

TABLE 1 Patient characteristics at baseline and day 7 plasma piperaquine concentrations for 93 randomly selected patients

Characteristic ^a	Value for patients			<i>P</i>
	Total (<i>n</i> = 425)	Western Cambodia (<i>n</i> = 147)	Eastern Cambodia (<i>n</i> = 278)	
No. (%) male	341 (80.2)	121 (82.3)	220 (79.1)	0.52 ^c
Wt (kg) (median [range])	50 (10–93)	53 (10–93)	49 (11–82)	<10 ^{-4d}
Age (yr) (median [range])	22 (3–58)	25 (3–58)	20 (3–58)	0.002 ^d
No. (%) of patients in the following age group:				
<5 yr old	4 (0.9)	1 (0.7)	3 (1.1)	0.06 ^e
5–14 yr old	77 (18.1)	18 (12.2)	59 (21.2)	
≥14 yr old	344 (80.9)	128 (87.1)	216 (77.7)	
Axillary temp (°C) (median [range])	38.7 (36.5–41.0)	38.5 (36.5–40.6)	38.7 (36.5–41.0)	0.16 ^d
Day 0 parasitemia (median [IQR] parasite count per μl)	23,212 (6,478–70,787)	17,822 (5,305–71,442)	26,275 (8,581–69,891)	0.10 ^d
Dose (mg/kg/day) (median [IQR])				
Dihydroartemisinin	2.4 (2.1–2.6)	2.3 (2.0–2.5)	2.4 (2.2–2.7)	0.0004 ^d
Piperaquine	18.8 (17.1–20.9)	18.1 (16.3–20.0)	19.2 (17.5–21.3)	0.0003 ^d
No. of patients with target doses of DHA (≥2 mg/kg/day) and piperaquine (≥16 mg/kg/day)/total no. of patients (%)	363/425 (85.4)	125/147 (85.0)	238/278 (85.6)	0.95 ^c
Day 7 data for randomly selected patients ^b				
Plasma piperaquine concn (ng/ml) (median [range])	41.8 (30.3–62.7)	48.3 (13.5–107.5)	38.7 (14.3–100.6)	0.18 ^d
No. (%) of patients with adequate plasma piperaquine concns (≥30 ng/ml)	72 (77)	49 (78)	23 (74)	0.80 ^c

^a IQR, interquartile range.^b Day 7 data were obtained for a subset of 93 patients, 62 from western Cambodia and 31 from eastern Cambodia.^c Calculated by Fisher's exact test.^d Calculated by the Mann-Whitney U test.^e Calculated by the chi-square test.

recurrent parasitemia was significantly higher in patients from western Cambodia (22/143; 15.4% [95% CI, 9.6% to 23.3%]) than in those from eastern Cambodia (7/276; 2.5% [95% CI, 1.0% to 5.2%]) ($P < 10^{-6}$). By PCR, all patients with recrudescences had recrudescent infections, observed at day 14 (1 patient with LPF), day 21 (4 with LCF and 1 with LPF), day 28 (3 with LCF and 5 with LPF), day 35 (7 with LCF and 2 with LPF), and day 42 (3 with LCF and 3 with LPF). The cumulative risk of DHA-PP treatment failure at day 42 was higher for western Cambodia patients, with a mean survival time of 40 days (95% CI, 39.1 to 40.9 days), than for eastern Cambodia patients (mean survival time, 41.8 days [95% CI, 41.7 to 42.0 days]) ($P < 10^{-6}$ by the log rank test) (Fig. 2). Among the 11 patients with recrudescent infections for whom day 7 plasma PP concentrations were measured, all but 3 (with 25.5, 29.1, and 29.8 ng/ml, respectively) had adequate concentrations (Fig. 3).

The PRR at 24 h (median, 94.0%) and 48 h (median, 100%) were significantly lower for patients from western Cambodia than for those from eastern Cambodia (86.8% versus 96.8% [$P < 10^{-5}$] and 99.0% versus 100% [$P < 10^{-4}$], respectively), while the proportion of parasite-positive patients on day 3 was significantly higher (31.2% versus 17.4% [$P = 0.002$]). All these clinical proxies of artemisinin resistance were concordant with the higher prevalence of K13 mutant alleles in western Cambodia (88.6% versus 33.6% in eastern Cambodia [$P < 10^{-14}$]). The C580Y allele was the most predominant (~86%) mutant allele (Table 2).

The median IC₅₀ for day 0 isolates successfully tested against PP (213/307 isolates [69%]) were similar in the two areas ($P =$

0.06), while for mefloquine (260/307 isolates [85%]), the median IC₅₀ for day 0 isolates from eastern Cambodia were significantly higher than those for western Cambodia (55.4 nM versus 37.7 nM; $P = 0.003$) (Table 2). However, the median copy numbers of the *Pfmdr-1* gene and the proportions of *Pfmdr-1* amplified day 0 isolates were similar in the two areas (P , 0.2 and 0.3, respectively). Finally, we found significant impacts of

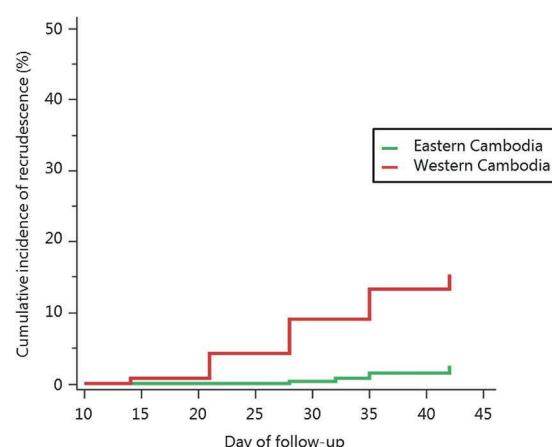


FIG 2 Cumulative risk at day 42 of patients with *P. falciparum* failing DHA-PP treatment. Shown is the overall difference between patients from western (red) and eastern (green) sites ($P = 0.01$). The hazard ratio (for western Cambodia versus eastern Cambodia) is 6.5 (95% CI, 2.9 to 14.1).

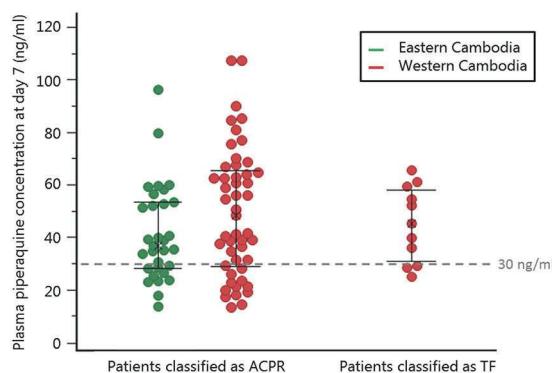


FIG 3 Plasma PP concentrations at day 7 as a function of clinical outcome. The gray dashed line indicates the PP concentration that defines adequate PP absorption (≥ 30 ng/ml). Red dots represent patients from western Cambodia sites, and green dots represent those from eastern Cambodia sites. ACPR, adequate clinical and parasitological response; TF, treatment failures.

Pfmdr-1 gene amplification on the median IC₅₀ both of mefloquine and of PP for day 0 isolates: the median IC₅₀ of mefloquine was 43.6 nM for isolates with a single copy of *Pfmdr-1* ($n = 204$) versus 95.1 nM for isolates with >1 *Pfmdr-1* copy ($n = 56$) ($P < 10^{-3}$), and the median IC₅₀ of PP was 48.0 nM for isolates with a single *Pfmdr-1* copy ($n = 173$) versus 40.0 nM for isolates with >1 *Pfmdr-1* copy ($n = 40$) ($P = 0.0004$).

The risk factors associated with DHA-PP treatment failure, presented in Table 3, were evaluated for the 93 patients for whom day 7 PP plasma concentrations were measured. Besides the residential location of the patients (western Cambodia) (OR = 14.1; 95% CI, 0.9 to 247; $P = 0.07$), the most significant risk factor associated with DHA-PP treatment failure was infection by parasites carrying the K13 mutant allele (OR = 17.5; 95% CI, 1 to 308; $P = 0.04$). The IC₅₀ levels for PP were independent of DHA-PP treatment outcomes (see Fig. S3 in the supplemental material), in contrast to the median mefloquine IC₅₀, which was found to be significantly lower for the isolates of patients with recrudescence infections (18.7 versus 39.7 nM; $P = 0.005$).

TABLE 2 Clinical responses to DHA-PP treatment (42-day follow-up) and parasitological parameters according to the study sites

Characteristic ^a	Value for patients		<i>P</i>
	Total ($n = 419$) ^b	Western Cambodia ($n = 143$)	
Clinical data			
PRR (median [range])			
At 24 h	13 (0.3–6,150)	7 (0.3–1,064)	$<10^{-10c}$
At 48 h	49 (2–16,302)	33 (2–1,368)	$<10^{-3c}$
No. (%) parasite positive on day 3	92 (22.1)	45 (31.2)	0.002 ^d
Parasitemia (median [range] parasite count per μ l)			
Day 1	813 (0–196,935)	2,680 (0–160,889)	$<10^{-9c}$
Day 2	0 (0–25,182)	182 (0–25,182)	$<10^{-10c}$
Day 3	0 (0–5,626)	0 (0–5,551)	$<10^{-3c}$
No. (%) with the following PCR-corrected outcome (per protocol):			
Treatment failure	29 (6.9)	22 (15.4)	$<10^{-6d}$
ACPR	390 (93.1)	121 (84.6)	$<10^{-5e}$
LCF	17 (4.0)	14 (9.8)	3 (1.1)
LPF	12 (2.9)	8 (5.6)	4 (1.4)
Parasitological parameters			
No. (%) with wild-type K13 allele	200 (47.7)	17 (11.4)	$<10^{-12d}$
No. (%) with mutant K13 alleles	219 (52.3)	126 (88.1)	$<10^{-7d}$
C580Y	188 (44.8)	124 (86.7)	$<10^{-12d}$
Y493H	26 (6.2)	1 (0.7)	$<10^{-3d}$
R539T	1 (0.2)	1 (0.7)	1 (0.4)
D584V	1 (0.2)	0	1 (0.4)
P553L	1 (0.2)	0	1 (0.4)
V568G	1 (0.2)	0	1 (0.4)
<i>Pfmdr-1</i> copy no. (median [range])	1 (1–4)	1 (1–4)	0.2 ^c
No. (%) of <i>P. falciparum</i> isolates with amplified <i>Pfmdr-1</i> gene	81 (19.4)	23 (16.2)	0.3 ^d
IC ₅₀ (nM) (median [range]) on day 0			
Piperazine	43.7 (4.4–77.8)	51.1 (29.2–71.1)	42.2 (4.3–77.8)
Mefloquine	51.7 (10.3–263.7)	37.7 (10.3–217.4)	55.4 (13.0–263.7)

^a PRR, parasite reduction ratios; ACPR, adequate clinical and parasitological response; LCF, late clinical failure; LPF, late parasitological failure; IC₅₀, 50% inhibitory concentration; *Pfmdr-1*, *Plasmodium falciparum* multidrug resistance gene 1.

^b Six patients were lost to follow-up, on days 3, 7, 14, 21 (2 patients), and 35.

^c Calculated by the Mann-Whitney U test.

^d Calculated by the Fisher exact test.

^e Calculated by the chi-square test.

TABLE 3 Human and parasitological risk factors associated with DHA-PP treatment failure

Risk factor ^a	Value for patients with:		<i>P</i>
	ACPR ^b (<i>n</i> = 82)	TF ^c (<i>n</i> = 11)	
Human			
Site (no. of patients in western/eastern Cambodia)	51/31	11/0	0.01 ^d
Age (yr) (median [range])	22.5 (3.0–58.0)	19.0 (9.0–45.0)	0.23 ^e
Sex (no. [%] male)	69 (84)	7 (64)	0.11 ^d
Wt (kg) (median [range])	51.5 (10.0–93.0)	45.0 (23.0–59.0)	0.30 ^e
Axillary temp (°C) (median [range])	38.5 (36.4–40.0)	38.5 (37.5–40.0)	0.52 ^e
Drug dose (mg/kg/day) (median [range])			
Dihydroartemisinin	2.3 (1.3–4.0)	2.4 (2.0–3.0)	0.38 ^e
PP	18.5 (10.3–32.0)	19.2 (16.3–24.0)	0.35 ^e
Target dose (no. [%] with ≥2 mg/kg/day DHA and ≥16 mg/kg/day PP)	71 (87)	11 (100)	0.35 ^d
Plasma PP concn at day 7 (ng/ml) (median [range])	41.3 (13.5–107.5)	50.7 (27.9–73.7)	0.39 ^e
Parasite positivity on day 3 (no. [%])	19 (23)	3 (27)	0.71 ^d
Parasitological			
Day 0 parasitemia (median [IQR] parasite count per μl)	39,001 (7,943–78,589)	7,938 (5,213–106,919)	0.60 ^e
Mutant K13 allele (no. [%])	46 (57)	11 (100)	0.006 ^d
Mutant K13 C580Y allele (no. [%])	43 (53)	11 (100)	0.002 ^d
<i>Pfmdr-1</i> copy no. (median [range])	1 (1–4)	1 (1)	0.1 ^e
Amplified <i>Pfmdr-1</i> gene (no. [%] of <i>P. falciparum</i> isolates)	17 (21)	0	0.20 ^d
IC ₅₀ (nM) (median [IQR]) at day 0			
PP	37.4 (32.8–43.2)	34.5 (27.4–38.4)	0.28 ^e
Mefloquine	39.7 (25.2–66.7)	18.7 (12.5–25.4)	0.005 ^e

^a IQR, interquartile range.^b ACPR, adequate clinical and parasitological response.^c TF, treatment failure (including LCF and LPF).^d Calculated by Fisher's exact test.^e Calculated by the Mann-Whitney U test.

DISCUSSION

This study has provided robust evidence that *P. falciparum* parasites resistant to PP are prevalent and are circulating in western Cambodia, an area where artemisinin resistance is very common (>85% of parasites have K13 mutations), confirming a recent report from Oddar Meancheay province (25). As a partner drug, PP must now be added to the growing list of failed drugs in a country where multidrug-resistant *P. falciparum* is becoming more challenging to treat with ACTs.

Although WHO therapeutic efficacy studies have been conducted for more than 15 years by the Cambodian National Malaria Control Program, plasma drug concentration data were not included routinely. Such data are essential for evaluation of antimalarial drug resistance according to the WHO definition, whereby a parasite can survive or multiply in the presence of therapeutic or tolerated supratherapeutic drug concentrations that are able to penetrate the parasitized red blood cell. We used a plasma PP concentration of ≥30 ng/ml at day 7 as the threshold value for determining adequate PP exposure. Indeed, it has been shown that this simplified measurement of exposure to PP is particularly suitable for long-half-life drugs and better than the total area under the curve (AUC) (31). In addition, several clinical studies have demonstrated that a day 7 plasma PP concentration of <30 ng/ml was associated with a higher risk of developing recurrent *P. falciparum* infections following DHA-PP treatment (27, 32, 33). In our study, we observed that the PP dose administered (in milligrams per kilogram) differed 3-fold (from 10 to 32 mg/kg/day) between patients and resulted in an 8-fold difference in plasma PP concen-

trations, but with no relationship between the two (see Fig. S2 in the supplemental material). Wide interindividual differences in plasma PP concentrations characterize the PP pharmacokinetics, and PP absorption is increased by fatty foods (34, 35). Among our patients with recrudescence infections, 8/11 had day 7 plasma PP concentrations of ≥30 ng/ml (ranging from 32 to 73 ng/ml), while 3 had PP concentrations just below the validated threshold (25.5, 29.1, and 29.8 ng/ml, respectively).

As in previous studies, *in vitro* PP data did not show a significant difference in the IC₅₀ between day 0 isolates collected from cured and failed patients, reconfirming the lack of correlation between *in vitro* and *in vivo* data. Of note, we observed that only 69% of the *in vitro* PP assays were able to provide interpretable IC₅₀ curves, while for mefloquine, IC₅₀ curves were interpretable for 85% of the isolates tested (*P* = 0.03). In addition, the proportion of uninterpretable IC₅₀ curves was significantly higher for isolates from patients with treatment failure than for isolates from cured patients only for PP (77.8% versus 21.8% [*P* < 10⁻⁸] for PP and 22.2% versus 12.1% for mefloquine). It is obvious that the absence of a reliable *in vitro* phenotype correlating with *in vivo* data and the absence of a molecular marker for PP resistance constitute a striking gap and result in a lack of potentially useful tools for the surveillance of PP resistance. More work on this is needed.

Western Cambodia has been the epicenter of multidrug-resistant *P. falciparum* for many years. Resistance has developed sequentially in response to the sequential use of antimalarial drugs; artemisinin resistance is the most recent addition. The rapid development of DHA-PP treatment failure in western Cambodia

appears to be highly correlated with the presence of K13 mutations and the increased sensitivity of *P. falciparum* isolates to mefloquine (36, 37). Interestingly, DHA-PP treatment failures have not been reported in other countries of the Greater Mekong Sub-region, such as Myanmar and Vietnam, despite high prevalence rates of K13 mutant alleles in these countries and many years of intense use (18, 19, 38, 39). However, in countries where artemisinin resistance is prevalent, we speculate that through the exposure of greater parasite biomasses to ACTs *in vivo*, artemisinin resistance could promote the evolution of resistance to partner drugs such as PP. Therefore, the notion that artemisinin resistance can lead to the emergence of partner drug resistance in ACTs needs to be further evaluated, since all ACTs failed in western Cambodia immediately after implementation, suggesting the emergence of partner drug resistance before or concomitantly with artemisinin resistance.

The antimalarial drug situation in Cambodia is deteriorating. As an immediate measure, policy makers in Cambodia, in conjunction with the WHO, decided in 2014 to replace DHA-PP with AS-MQ in areas where DHA-PP is failing and where parasites have low amplified *Pfmdr-1* copy numbers. However, new therapeutic strategies, such as the use of triple ACTs, are urgently needed to eliminate multidrug-resistant malaria parasites in western Cambodia and must be tested.

ACKNOWLEDGMENTS

We thank the patients for agreeing to take part in these studies. We are also grateful to the provincial health directors and the local health staff at the study sites.

This work was supported by the Institut Pasteur du Cambodge and by USAID and BMGF grants through the WHO. W.R.J.T. was supported (from October 2013 to October 2014) by France Expertise International under the 5% initiative.

We declare that no conflicts of interest exist. The views expressed in this article are those of the authors. D.M.B. and P.R. are staff members of the WHO. The authors alone are responsible for the views expressed in this publication, and these views do not necessarily represent the decisions, policy, or views of the WHO.

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Chapitre V. Développement d'un nouveau test phénotypique pour la détection de la résistance à la PPQ chez *P. falciparum*

1. Introduction

Comme nous l'avons vu avec l'article précédent ⁹², les études récemment conduites au Cambodge rapportent des taux d'échecs cliniques allant de 15 à 60% après un traitement standard de 3 jours de DHA-PPQ, l'ACT recommandé dans ce pays depuis 2008. Ces résultats alarmants indiquent que des parasites ayant une susceptibilité réduite à la fois à l'artémisinine et à la pipéraquine sont maintenant prévalents à l'ouest du Cambodge et dans les provinces alentours. La résistance à l'ART est aujourd'hui clairement définie, avec un phénotype clinique (clairance parasitaire retardée ou persistance de parasites microscopiquement détectables à J3) ⁴⁹⁻⁵², un phénotype *in vitro* (Ring-stage Survival Assay ou RSA) ⁵⁵ et un marqueur moléculaire (mutations dans le domaine en hélice du gène *K13*) ⁸⁶, comme nous l'avons présenté dans le premier chapitre de cette thèse. En revanche, la résistance à la PPQ demeure mal caractérisée, puisqu'elle ne peut actuellement être détectée que par l'identification des échecs de traitements tardifs chez les patients traités avec un protocole standard de 3 jours de DHA-PPQ. Il n'existe donc aucune preuve solide de résistance parasitaire à la PPQ, puisqu'il n'y a ni phénotype *in vitro* fiable ni marqueur moléculaire validé pour la résistance à la PPQ.

Puisqu'aucune association directe n'a été trouvée entre des valeurs élevées d'IC₅₀ ou d'IC₉₀ à la PPQ et les échecs de traitements, nous avons développé un nouveau test *in vitro*, le Piperaquine Survival Assay (PSA), reposant sur le même principe que le RSA ⁵⁵ : les parasites sont exposés à des doses pharmacologiquement pertinentes de PPQ (200nM durant 48h), puis la susceptibilité des parasites à la drogue est évaluée à 72h par microscopie, en estimant la proportion de parasites viables qui ont pu se développer en rings et trophozoites

de deuxième génération. Dans une étude rétrospective, nous avons évalué l'association entre la survenue d'une recrudescence et le taux de survie en PSA *in vitro* (parasites synchronisés au stade « jeunes rings » 0-3h) de 32 isolats adaptés en culture et porteurs de la mutation *K13* C580Y (donc résistants à l'artémisinine), collectés à l'enrôlement de patients traités en 2012 avec 3 jours de DHA-PPQ. Puis dans une étude prospective menée en 2014, nous avons examiné si le taux de survie en PSA *ex vivo* combiné au polymorphisme de *K13* permettait de prédire un échec de traitement à la DHA-PPQ. Enfin, nous avons souhaité vérifier l'association de marqueurs moléculaires candidats pour la résistance à la PPQ (notamment *Pfmdr1*) avec nos valeurs de PSA *in vitro*.

2. Résultats et discussion

Les médianes des taux de survies de PSA étaient plus élevées pour les isolats J0 de patients recrudescents que pour ceux de patients guéris, et ce dans les deux études : 51.9% contre 34.4%, $P = 0.04$ dans l'étude rétrospective (PSA *in vitro*) et 39.2% contre 0.17%, $P < 10^{-7}$ dans l'étude prospective (PSA *ex vivo*). De plus, tous les isolats provenant de patients recrudescents avaient un taux de survie en PSA (*in vitro* ou *ex vivo*) $\geq 10\%$, qui semble donc être une valeur seuil pertinente pour la résistance à la PPQ.

L'étude rétrospective, menée uniquement sur des parasites porteurs de la mutation *K13* C580Y, nous a permis de montrer que la résistance à la PPQ n'était pas directement liée aux polymorphismes du gène *K13*, puisque 8 des 21 isolats collectés chez des patients non-recrudescents se sont révélés susceptibles à la PPQ (PSA $< 10\%$). En revanche, l'étude prospective nous a appris que la quasi-totalité des parasites présentant un taux de survie en PSA $\geq 10\%$ étaient également porteur d'une mutation *K13*. Cela reflète probablement le fait qu'*in vivo*, la résistance à la pipéraquine est sélectionnée chez des parasites capables de résister d'abord à l'action rapide de l'artémisinine pour pouvoir résister ensuite à l'action

longue durée de la drogue partenaire. Ainsi, les patients infectés avec des parasites porteurs d'une mutation *K13* et avec un taux de survie en PSA *ex vivo* $\geq 10\%$ étaient exposés à un risque 32 fois plus élevé de faire une recrudescence (95% CI, 4.5-224 ; $P = 0.0005$).

Enfin, nous avons également pu confirmer que la résistance à la PPQ était associée à la présence d'une unique copie du gène *Pfmdr1*, et donc à des valeurs basses d'IC₅₀ à la MQ : en effet, l'ensemble des parasites présentant un taux de survie en PSA $\geq 10\%$ n'étaient porteurs que d'une copie du gène *Pfmdr1* (médiane des taux de survie en PSA : 48%, contre 0.6% pour les parasites porteurs de plusieurs copies, $P = 0.0001$). La réciproque n'est cependant pas vérifiée, puisque certains parasites porteurs d'une seule copie de *Pfmdr1* étaient susceptibles à la PPQ en test PSA (taux de survie $< 10\%$). Ces données appuient fortement la récente recommandation des programmes nationaux de contrôle du paludisme au Cambodge d'utiliser l'association ATS-MQ dans les provinces où le taux d'échecs à la DHA-PPQ dépasse 10%.

En conclusion, notre travail a démontré que le PSA était capable de capturer efficacement le phénotype de résistance à la PPQ, ouvrant ainsi la voie à l'identification de marqueurs moléculaires et à l'évaluation de l'efficacité de molécules alternatives. De plus, la combinaison du PSA *ex vivo* et du génotypage *K13* permet une surveillance efficace et accessible des résistances à l'artémisinine et à la pipéraquine dans les régions utilisant l'association DHA-PPQ.

3. Article 5

***Plasmodium falciparum* dihydroartemisinin-piperaquine failures in Cambodia are associated with mutant K13 parasites presenting high survival rates in novel piperaquine *in vitro* assays: retrospective and prospective investigations.**

Duru V, Khim N, Leang R, Kim S, Domergue A, Kloeung N, Ke S, Chy S, Eam R, Khean C, Loch K, Ken M, Lek D, Beghain J, Ariey F, Guerin PJ, Huy R, Mercereau-Puijalon O, Witkowski B, Menard D.

BMC Med. 2015 Dec 22;13:305. doi: 10.1186/s12916-015-0539-5.

RESEARCH ARTICLE

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Plasmodium falciparum dihydroartemisinin-piperaquine failures in Cambodia are associated with mutant K13 parasites presenting high survival rates in novel piperaquine in vitro assays: retrospective and prospective investigations

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Abstract

Background: The declining efficacy of dihydroartemisinin-piperaquine against *Plasmodium falciparum* in Cambodia, along with increasing numbers of recrudescent cases, suggests resistance to both artemisinin and piperaquine. Available in vitro piperaquine susceptibility assays do not correlate with treatment outcome. A novel assay using a pharmacologically relevant piperaquine dose/time exposure was designed and its relevance explored in retrospective and prospective studies.

Methods: The piperaquine survival assay (PSA) exposed parasites to 200 nM piperaquine for 48 hours and monitored survival 24 hours later. The retrospective study tested 32 culture-adapted, C580Y-K13 mutant parasites collected at enrolment from patients treated with a 3-day course of dihydroartemisinin-piperaquine and having presented or not with a recrudescence at day 42 (registered ACTRN12615000793516). The prospective study assessed ex vivo PSA survival rate alongside K13 polymorphism of isolates collected from patients enrolled in an open-label study with dihydroartemisinin-piperaquine for uncomplicated *P. falciparum* malaria in Cambodia (registered ACTRN12615000696594).

Results: All parasites from recrudescent cases had in vitro or ex vivo PSA survival rates $\geq 10\%$, a relevant cut-off value for piperaquine-resistance. Ex vivo PSA survival rates were higher for recrudescent than non-recrudescent cases (39.2 % vs. 0.17 %, $P < 1 \times 10^{-7}$). Artemisinin-resistant K13 mutants with ex vivo PSA survival rates $\geq 10\%$ were associated with 32-fold higher risk of recrudescence (95 % CI, 4.5–224; $P = 0.0005$).

Conclusion: PSA adequately captures the piperaquine resistance/recrudescence phenotype, a mainstay to identify molecular marker(s) and evaluate efficacy of alternative drugs. Combined ex vivo PSA and K13 genotyping provides a convenient monitor for both artemisinin and piperaquine resistance where dihydroartemisinin-piperaquine is used.

Keywords: Artemisinin combination therapies, Artemisinin resistance, Cambodia, Ex vivo testing, Falciparum, In-vitro testing, Malaria, Piperaquine resistance, Treatment failure

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Background

Artemisinin combination therapies (ACTs), the most effective antimalarial medicines, are the mainstay of the management of uncomplicated *Plasmodium falciparum* malaria in endemic countries [1]. Over the last decade, their wide use has contributed to a reduction in the worldwide burden of malaria [1, 2]. Unfortunately, the recent emergence of *P. falciparum* resistance to artemisinin derivatives in Southeast Asia challenges malaria control and elimination efforts. Artemisinin-resistant *P. falciparum* malaria, first reported in western Cambodia in 2008–2009 [3, 4], has since been observed in Thailand, Myanmar, Vietnam, and Lao People's Democratic Republic [5–9], as well as China [10]. Although parasites are resistant to artemisinin derivatives [11] resulting in delayed parasite clearance, ACTs remain clinically and parasitologically efficacious thanks to partner drug efficacy [9]. However, recent studies in Cambodia reported a 15–60 % rate of late clinical failures after the standard 3-day course of dihydroartemisinin-piperaquine, the recommended ACT since 2008 [12–16]. This indicates that parasites with reduced susceptibility to both artemisinin and piperaquine are now prevalent in western Cambodia and neighboring provinces.

Artemisinin resistance is currently clinically defined as a parasite clearance half-life of 5 hours or more in Southeast Asia or persistence of microscopically detectable parasites on day 3 after treatment with an ACT [9]. The corresponding in vitro phenotype is a survival rate of more than 1 % in the Ring-stage Survival Assays (in vitro RSA^{0–3h} and ex vivo RSA) [11, 17] associated with polymorphisms in the propeller domain of the *Kelch 13* gene [17–19]. In contrast, piperaquine resistance is poorly characterized. It is currently identified by late clinical failures in patients treated with standard 3-day course of dihydroartemisinin-piperaquine. However, robust evidence of parasite-dependent resistance to piperaquine is lacking as there is no reliable in vitro phenotype and no validated genetic molecular marker. Population-based analysis of in vitro susceptibility showed recent temporal increasing geometric means of inhibitory concentration 50 % (IC_{50}) for piperaquine [12, 13, 20], but a demonstration of a direct association between high piperaquine IC_{50} or inhibitory concentration 90 % (IC_{90}) for isolates prior to treatment and dihydroartemisinin-piperaquine failure is lacking. Moreover, IC_{50} or IC_{90} for piperaquine in day 0 isolates from recrudescence patients are distributed over a wide range, which overlaps with IC_{50} values of isolates from non-recrudescence patients. In other words, whether the temporal increase of geometric mean of IC_{50} or IC_{90} for piperaquine reflects elimination of the most susceptible parasites to piperaquine or emergence of piperaquine-resistant parasites remains unknown.

Herein, we report a novel in vitro assay – the piperaquine survival assay (PSA) – designed to mimic in vivo exposure of parasites to a pharmacologically relevant dose of piperaquine (200 nM for 48-hours as the piperaquine half-life is estimated to ~9 days) [21]. In a retrospective study, we evaluate the association between occurrence of recrudescence and in vitro PSA survival rates of C580Y K13-mutant (artemisinin-resistant), culture-adapted *P. falciparum* isolates collected at day 0 from patients treated in 2012 with dihydroartemisinin-piperaquine. In this prospective study, conducted in 2014, we explore whether the survival rate in the ex vivo PSA combined with K13 polymorphism are predictive of dihydroartemisinin-piperaquine treatment failure. Candidate molecular markers reported as associated with piperaquine-resistant *P. falciparum* are explored in parasites classified as piperaquine-resistant or piperaquine-susceptible by the in vitro PSA assay.

Methods

Study design and patients

Retrospective investigation

One hundred forty six patients with acute uncomplicated falciparum malaria were enrolled in WHO therapeutic efficacy studies conducted in 2012–2013 at health centres in western and eastern Cambodia [16]. After obtaining written informed consent from patients or parents/guardians of children, blood samples were collected before treatment into acid-citrate-dextrose tubes (Becton-Dickinson, Franklin Lakes, NJ, USA) and were then adapted to culture and in vitro susceptibility testing [11]. Patients were treated with dihydroartemisinin-piperaquine (Duo-Cotexin®, dihydroartemisinin 40 mg and piperaquine 320 mg, Zhejiang Holley Nanhu Pharmaceutical Co. Ltd, Jiaxing, Zhejiang province, China) and followed-up for 42 days (2009 WHO protocol) [16]. The proportion of *P. falciparum* recrudescent infections at day 42, after PCR-correction, was assessed, along with blood piperaquine concentrations at day 7 [16].

Ethical approvals were obtained from the National Ethical Committee for Health Research of the Cambodian Ministry of Health. The trial was registered at the Australian New Zealand Clinical Trials Registry (ACTRN12615000793516). Among these patients, 32 culture-adapted parasites harboring the artemisinin-resistance C580Y K13 mutation were chosen for testing (to assess only piperaquine resistance): 21 from non-recrudescent and 11 from recrudescent patients (Additional file 1) [16].

Prospective study

Between May 2014 and February 2015, patients with uncomplicated falciparum malaria were recruited, treated

and followed-up for 42 days at district health centres in Rattanakiri, Siem Reap, Stung Treng, and Mondulkiri provinces [16]. Briefly, after obtaining written informed consent from patients or parents/guardians of children, a finger prick blood sample was collected at enrolment for thick/thin blood films and parasite genotyping and a 5 mL venous blood sample was collected into acid-citrate-dextrose tubes for ex vivo PSA. *Falciparum* malaria was diagnosed by microscopic examination of Giemsa-stained thick/thin blood films and parasitemia was calculated from the number of parasites per 200 white blood cells, assuming a total white cell count of 8000/ μ L [16]. Patients failing dihydroartemisinin-piperaquine therapy with recurrent *P. falciparum* infection were retreated with artemether plus mefloquine as per national guidelines. Filter-paper blood spots collected on day 0 and day of recurrent parasitemia were used to determine 12 single-nucleotide polymorphisms [11] and classify recurrent infections into reinfections in case of distinct genetic profiles or recrudescence parasites when those profiles were similar. The primary outcome was PCR-corrected *P. falciparum* recrudescence within 42 days (see patient information in Additional file 2). Ethical approvals were obtained from the National Ethical Committee for Health Research of the Cambodian Ministry of Health and the trial was registered at Australian New Zealand Clinical Trials Registry (ACTRN12615000696594).

In vitro parasite adaptation

Isolates collected on day 0 of enrolment were adapted to in vitro culture and maintained using the following conditions: 2 % hematocrit (O^+ blood group, blood bank, Phnom Penh, Cambodia) in RPMI 1640 supplemented with 2.5 % decomplemented human plasma (blood bank, Phnom Penh, Cambodia) and 0.5 % Albumax II (Gibco-Life Technologies SAS, France) at 37 °C in a 5 % CO₂, 5 % O₂ wet atmosphere [11]. Culture adaptation was considered successful after 3 weeks of culture. The 3D7 reference strain obtained from MR4 was maintained in the same conditions.

Standard isotopic in vitro susceptibility testing

Piperaquine, mefloquine, dihydroartemisinin and chloroquine were obtained from the WorldWide Antimalarial Resistance Network. In vitro susceptibility of culture-adapted *P. falciparum* parasites was assessed using the 48-h isotopic test monitoring incorporation of [³H]-hypoxanthine (Amersham, Les Ulis, France), as previously described [11], with the 3D7 line as a quality control. Results were expressed as IC₅₀ and IC₉₀, which values were determined by non-linear regression using the on-line WorldWide Antimalarial Resistance Network

IVART software and the on-line ICestimator software (www.antimalarial-icestimator.net), respectively [22].

Piperaquine survival assays (PSA)

The PSA were performed with 0–3-h post-invasion rings from culture-adapted parasites (in vitro PSA) or directly with parasites collected from patients (ex vivo PSA) (Fig. 1). Parasite density and hematocrit levels were adjusted to 0.1–2 % and 2 %, respectively. Parasites were cultivated for 48 h at 37°C under a 5 % CO₂, 5 % O₂ wet atmosphere with 200 nM piperaquine tetraphosphate tetrahydrate (exposed culture) or 0.5 % lactic acid (non-exposed culture). After 48 h, cultures were washed once with 12 mL RPMI 1640, resuspended in complete medium (RPMI 1640, 0.5 % Albumax II, 2 % heat-inactivated O+ plasma, 50 μ g/mL gentamicin), and cultured for a further 24 h. Thin blood smears were prepared, methanol-fixed and stained with 10 % Giemsa (Merck KGaA, Darmstadt, Germany) for 45 min. The proportion of viable parasites in exposed and non-exposed cultures was evaluated by counting parasites having developed into second-generation rings or trophozoites with normal morphology. For each assay, 20,000 erythrocytes were assessed by two independent microscopists blinded to the clinical data. In case of a difference greater than 20 %, slides were examined by a third microscopist, also blinded to the clinical data. Susceptibility to piperaquine was defined as the median survival rate calculated using the following formula:

$$\text{PSA survival rate (\%)} = \frac{\text{Number of viable parasites in exposed culture}}{\text{Number of viable parasites in non-exposed culture}} \times 100.$$

Detection of mutations and copy number variation of candidate resistant genes

Following gDNA extraction from the 32 culture-adapted parasites (QIAamp DNA Blood Mini Kit, Qiagen, Valencia, CA), whole-genome sequencing was performed using Illumina paired-reads sequencing [17]. Raw sequence files were filtered using FastQC tool and the trimmed reads from controlled Fastq files were mapped on the *P. falciparum* 3D7 reference genome with the Burrows-Wheeler Alignment. A pileup file was prepared using Samtools and formatted using in-house software to implement the data into the Wholegenome Data Manager database [17], which was used to align partial or whole genomes and detect mutations or copy number variation in PF3D7_0709000 [23], PF3D7_0523000 [16, 24], PF3D7_0112200 [25], PF3D7_1229100 [26], MAL10:688956 [13], MAL13:1718319 [13], PFE1010w [23], and PFE1085w [23] (Table 1).

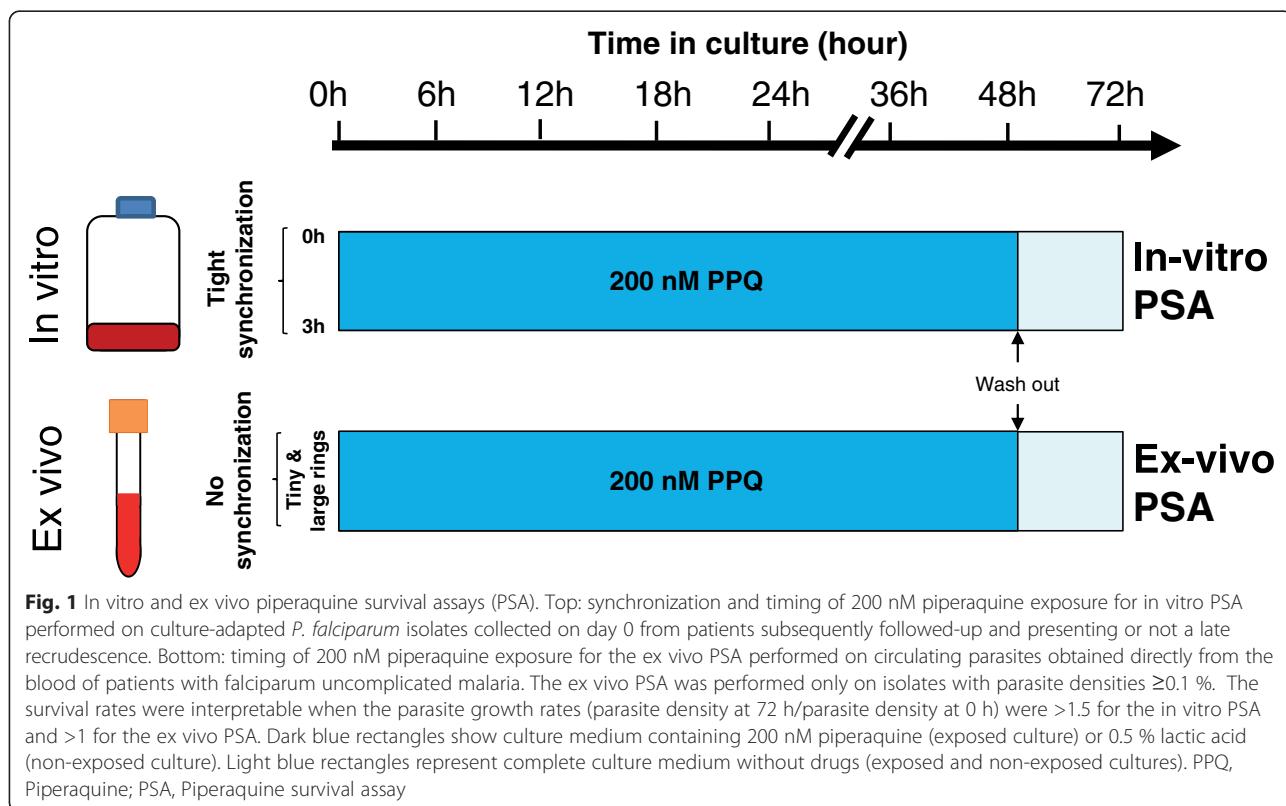


Fig. 1 In vitro and ex vivo piperaquine survival assays (PSA). Top: synchronization and timing of 200 nM piperaquine exposure for in vitro PSA performed on culture-adapted *P. falciparum* isolates collected on day 0 from patients subsequently followed-up and presenting or not a late recrudescence. Bottom: timing of 200 nM piperaquine exposure for the ex vivo PSA performed on circulating parasites obtained directly from the blood of patients with falciparum uncomplicated malaria. The ex vivo PSA was performed only on isolates with parasite densities $\geq 0.1\%$. The survival rates were interpretable when the parasite growth rates (parasite density at 72 h/parasite density at 0 h) were > 1.5 for the in vitro PSA and > 1 for the ex vivo PSA. Dark blue rectangles show culture medium containing 200 nM piperaquine (exposed culture) or 0.5 % lactic acid (non-exposed culture). Light blue rectangles represent complete culture medium without drugs (exposed and non-exposed cultures). PPQ, Piperaquine; PSA, Piperaquine survival assay

DNA from day 0 blood samples (2014-projective study) was used to genotype the K13-propeller domain (PF3D7_1343700) and measure *P. falciparum multidrug resistance 1* copy number, as previously described [16].

Statistical analysis

Data were analyzed with Microsoft Excel and MedCalc version 12 (Mariakerke, Belgium). Quantitative and qualitative data were expressed as median (interquartile range, IQR) or proportion (%), respectively. The Mann-Whitney U or the Kruskal-Wallis (H-test) tests were used for non-parametric comparisons. For categorical variables, proportions were examined by χ^2 or by Fisher exact tests. Relative risks were estimated using the Mantel-Haenszel test. The cumulative risk of failure at day 42 was assessed by survival analysis with the Kaplan-Meier method. Treatment outcome between patients harboring mutant or wild-type K13 parasites and PSA ex vivo survival rate $<$ or $\geq 10\%$ at day 0 were compared using the Mantel-Haenszel log rank test and hazard ratio (HR). Two sided *P* values of <0.05 were considered statistically significant.

Results

In vitro phenotype of piperaquine resistance: 2012 retrospective analysis

Investigations of various risk factors associated with dihydroartemisinin-piperaquine failure in 32 artemisinin-

resistant culture-adapted parasites collected at enrollment showed that the only significant parameter was the median mefloquine IC₅₀, which was significantly higher in isolates of non-recrudescent compared to recrudescent patients (32.2 nM, IQR: 19.4–39.8 nM vs. 19.7 nM, IQR: 15.5–22.2 nM, respectively, *P* = 0.03) (Table 2).

Overall, we observed a lower frequency of interpretable curves for piperaquine (12/32, 37 %) compared to the other drugs tested (28/32; 87 % for mefloquine and 31/32, 97 % for chloroquine and dihydroartemisinin). A paradoxical increase of incorporation at high drug concentrations (≥ 100 –200 nM piperaquine) was repeatedly observed (Additional file 3), and this was more frequent in isolates from recrudescent (9/11, 82 %) than from non-recrudescent patients (11/21, 52 %). In contrast, every culture-adapted isolate gave interpretable in vitro PSA data and 83 % (19/23) of the isolates with a PSA survival rate $\geq 10\%$ had an unreliable concentration-response curve for piperaquine. Median survival rates were higher in day 0 isolates from recrudescent (51.9 %, IQR: 40.7–61.5 %) than non-recrudescent patients (34.4 %, IQR: 0.8–52.2 %, *P* = 0.04, Table 2). All day 0 culture-adapted isolates from recrudescent patients had a PSA survival rate $\geq 10\%$, whereas survival rates from non-recrudescent patients ranged from 0.3 % to 77.4 % (Fig. 2).

Table 1 Association between candidate molecular markers (mutations and copy number variation (CNV)) previously associated with piperaquine resistance and in vitro piperaquine survival assay (PSA) phenotypes of 32 culture-adapted isolates collected from patients treated with 3-day courses of dihydroartemisinin-piperaquine in 2012

Gene polymorphism	Piperaquine survival assay (PSA)					
	n	Survival rate (median, IQR)	P value [*]	PSA <10 % n = 8	PSA ≥10 % n = 21	P value ^{**}
<i>P. falciparum chloroquine resistant transporter gene - PfCRT (PF3D7_0709000)</i>						
Alleles	74I/75E/76 T/220S/271E/326S/343L/356 T/371I	7	61.5 (53.6–69.2)	0.005	0 (0 %)	7 (33 %)
	74I/75E/76 T/220S/271E/326S/356T/371I (Dd2 allele)	16	12.8 (0.6–43.7)		8 (100 %)	8 (38 %)
	74I/75E/76 T/220S/271E/326S/353V/356 T/371I	2	49.2 (37–61.5)		0 (0 %)	2 (9.5 %)
	74I/75E/76 T/97Y/220S/271E/326S/356T/371I	4	47.1 (40.9–61.2)		0 (0 %)	4 (19 %)
<i>P. falciparum multidrug resistance 1 gene - PfMDR-1 (PF3D7_0523000)</i>						
Alleles	643D	1	70.6	0.16	0 (0 %)	1 (5 %)
	184 F	16	51.8 (3.5–61.5)		5 (63 %)	11 (52 %)
	184 F/293D	3	19.3 (9.3–35.4)		1 (12 %)	2 (9.5 %)
	184 F/1087 L	6	44 (40.1–49.6)		0 (0 %)	6 (29 %)
	wild-type	3	0.8 (0.5–27.9)		2 (25 %)	1 (5 %)
CNV (957885 to 962144)	single copy	24	48.1 (38.2–60.1)	0.001	3 (38 %)	21 (100 %)
	multi-copy (184 F, n = 2 and wild-type, n = 3)	5	0.6 (0.4–2.1)		5 (62 %)	0 (0 %)
<i>P. falciparum multidrug resistance-associated protein 1 - PfmRP-1 (PF3D7_0112200)</i>						
Alleles	191Y/437A/785 N/876 V/1007 M	3	0.8 (0.5–83.1)	0.15	2 (25 %)	1 (5 %)
	191Y/437A/876 V	1	37		0 (0 %)	1 (5 %)
	191Y/437A/876 V/1390 T	16	50.5 (39.7–60.1)		1 (13 %)	15 (72 %)
	191Y/437A/876 V/1390 T/1669D	1	0.6		1 (12 %)	0 (0 %)
	191Y/325S/437A	8	22.9 (0.6–47.1)		4 (50 %)	4 (19 %)
<i>P. falciparum multidrug resistance-associated protein 2 - PfmRP-2 (PF3D7_1229100)</i>						
Alleles	199 V/295R/593D/714I/1527 T/1531I	1	0.7	0.07	1 (12.5 %)	0 (0 %)
	199 V/622D/646D/714I/1188 N/1527 T/1531I	2	3.5 (0.6–6.4)		2 (25 %)	0 (0 %)
	199 V/646D/714I/1176 N/1188 N/1527 T/1531I	1	0.8		1 (12.5 %)	0 (0 %)
	199 V/646D/714I/1188 N/1527 T/1531I	10	58.7 (29.0–62.1)		2 (25 %)	8 (35 %)
	199 V/646D/714I/1527 T/1531I	16	41.7 (38.2–51.6)		1 (12.5 %)	15 (65 %)
	199 V/646D/714I/964D/970 N/1527 T/1531I	1	0.3		1 (12.5 %)	0 (0 %)
MAL10:688956 (Chr10)						
Alleles	3D7 allele type (T)	10	22.9 (0.6–51.8)	0.20	5 (62.5 %)	5 (24 %)
	V1/S mutant-type (A)	19	46.7 (31–58.2)		3 (37.5 %)	16 (76 %)
MAL13:1718319 (Chr13)						
Alleles	3D7 allele type (A)	1	0.3	–	1 (12.5 %)	0 (0 %)
	V1/S mutant-type (T)	28	41.6 (12.8–57.7)		7 (87.5 %)	21 (100 %)
PFE1010w (Chr5, 831614 to 834340)						
CNV	single copy	29	–	–	8 (100 %)	21 (100 %)
	multi-copy	0	–		0 (0 %)	0 (0 %)

Table 1 Association between candidate molecular markers (mutations and copy number variation (CNV)) previously associated with piperaquine resistance and in vitro piperaquine survival assay (PSA) phenotypes of 32 culture-adapted isolates collected from patients treated with 3-day courses of dihydroartemisinin-piperaquine in 2012 (Continued)

PFE1085w (Chr5, 882373 to 884898)						
CNV	single copy	29	–	–	8 (100 %)	21 (100 %)
	multi-copy	0	–	–	0 (0 %)	0 (0 %)

Pfcrt Dd2, MAL10:688956 and MAL13:1718319 allele types are defined based on sequences available at plasmoDB.org

*Calculated by the Mann-Whitney U or Kruskal-Wallis (H-test) tests

**Calculated by the Fisher exact or χ^2 tests

***Significant P values are shown in bold font

Ex vivo PSA: 2014 prospective study

In a prospective study conducted in 2014, 178 patients presenting to district health centres with uncomplicated falciparum malaria were enrolled, administered standard 3-day dihydroartemisinin-piperaquine treatment

and followed-up to day 42 or day of failure [16]. Detection of K13-propeller mutations and ex vivo PSA survival rates of day 0 isolates were achieved for 74 patients (Additional file 4): 50 patients were classified as non-recrudescent and 24 patients as recrudescent,

Table 2 Patient and parasitological characteristics of 32 culture-adapted isolates and their association with dihydroartemisinin-piperaquine treatment outcome at day 42 in Cambodian patients, Cambodia (2012 retrospective study)

Risk factors	All patients n = 32	Non-recrudescent patients n = 21	Recrudescent patients n = 11	P value
Patient				
Age, years (median, IQR)	19 (16.5–23)	19 (17.2–22.2)	19 (14.2–23.5)	0.67*
Sex, male (n, %)	21 (66 %)	13 (62 %)	8 (73 %)	0.70**
Weight, kg (median, IQR)	49.5 (41.5–55.0)	50 (42.7–55.2)	45.0 (40.2–54.7)	0.49*
Axillary temperature, °C (median, IQR)	38.5 (38.1–39.5)	38.5 (38.4–39.5)	38.5 (38.1–39.4)	0.87*
Dihydroartemisinin dose, mg/kg/day (median, IQR)	2.4 (2.2–2.7)	2.4 (2.2–2.6)	2.4 (2.2–2.7)	0.53*
Piperaquine dose, mg/kg/day (median, IQR)	19.2 (17.5–21.3)	18.8 (17.4–21.0)	19.4 (17.6–21.7)	0.47*
Target dose ≥2/16 mg/kg/d DHA/PPQ (n, %)	29 (91 %)	18 (86 %)	11 (100 %)	0.53**
Day 7 plasma piperaquine concentration, ng/mL (median, IQR)	41.8 (31.6–58.5)	40.1 (30.4–56.7)	47.5 (32.7–60.4)	0.53*
Day 3 parasite positive (n, %)	10 (31 %)	6 (29 %)	4 (36 %)	0.70**
Parasite				
Day 0 N° parasites per μL (median, IQR)	15,879 (5,961–64,291)	13,936 (6,960–47,097)	28,455 (5,250–81,063)	0.59*
Presence of K13 C580Y allele (n, %)	32 (100 %)	21 (100 %)	11 (100 %)	1**
Pfmdr1 copy number (median, range)	1 (1–3)	1 (1–3)	1 (1)	0.11*
Chloroquine IC ₅₀ at D0 (nM)	Interpretable IC ₅₀ (n, %) median, IQR	31 (97 %) 183 (94–308)	20 (95 %) 199 (72–306)	11 (100 %) 150 (126–389)
Piperaquine IC ₅₀ at D0 (nM)	Interpretable IC ₅₀ (n, %) median, IQR	12 (37 %) 42.9 (22.4–52.7)	10 (48 %) 40.3 (7.6–52.1)	2 (18 %) 55.6
Piperaquine IC ₉₀ at D0 (nM)	Interpretable IC ₉₀ (n, %) median, IQR	12 (37 %) 81.7 (58.1–119.8)	10 (48 %) 72.2 (54.3–96.0)	2 (18 %) 132.6
Mefloquine IC ₅₀ at D0 (nM)	median, IQR	42.9 (22.4–52.7)	40.3 (7.6–52.1)	55.6
	median, IQR	24.4 (18.4–34.3)	32.2 (19.4–39.8)	19.7 (15.5–22.2)
DHA IC ₅₀ at D0 (nM)	Interpretable IC ₅₀ (n, %) median, IQR	31 (97 %) 0.86 (0.61–1.58)	20 (95 %) 0.88 (0.63–1.86)	11 (100 %) 0.83 (0.61–1.02)
Survival rate (RSA ^{0–3^h}) (n, %)	Interpretable (n, %) median, IQR	32 (100 %) 13.2 (9.9–18.1)	21 (100 %) 12.3 (10.0–15.2)	11 (100 %) 15.2 (8.1–20.9)
				0.54*

*Calculated by the Mann-Whitney U test

**Calculated by Fisher exact test

***Significant P values

with a mean time to recrudescence of 28 days (IQR: 21–32.5 days).

Recrudescent infections were strongly associated with day 0 parasites presenting high survival rates in the ex vivo PSA (median = 39.2 %, IQR: 24.5–57.6 %) contrasting with parasites from non-recrudescent patients that had uniformly low survival (median = 0.17 %, IQR: 0.15–0.59 %, $P < 1 \times 10^{-11}$) (Fig. 2). All day 0 blood samples from recrudescent patients had PSA survival rates $\geq 10\%$. Recrudescence was also associated with presence of a mutant K13 allele (C580Y or Y493H) on day 0 (8/50, 16 % in non-recrudescent patients vs. 23/24, 96 % in recrudescent patients, $P < 1 \times 10^{-10}$). Thus, artemisinin resistance (defined as presence of a mutant K13 allele) was strongly associated with piperaquine resistance (defined as PSA survival rate $\geq 10\%$; $P < 1 \times 10^{-14}$) (Fig. 2 and Additional file 2).

The cumulative incidence of parasitological failure after the 3-day dihydroartemisinin-piperaquine treatment was

significantly higher in patients infected by mutant K13 parasites with a PSA survival rate $\geq 10\%$ ($P < 1 \times 10^{-10}$, log rank test, Hazard Ratio = 14.3, 95 % CI, 4.6–44.6; Fig. 3). These patients had a 32-fold higher risk of recrudescence (95 % CI, 4.5–224; $P = 0.0005$); only 25.8 % (SD = 7.9 %) of these patients remained without parasites at day 42 after treatment.

Of note, as observed previously for artemisinin resistance [11], data from the prospective study show a progressive decrease in piperaquine-resistant *P. falciparum* parasites from Western to Eastern Cambodia: 88.2 % (15/17) in Siem Reap, 61.5 % (8/13) in Stung Treng to 25.0 % (4/16) and 21.4 % (6/28) in Mondulkiri and Rattanakiri, respectively.

Candidate molecular markers associated with piperaquine resistance

The 32 C580Y-K13 culture-adapted parasites were analyzed for possible association between candidate molecular markers and in vitro PSA survival rates (Table 1). Of

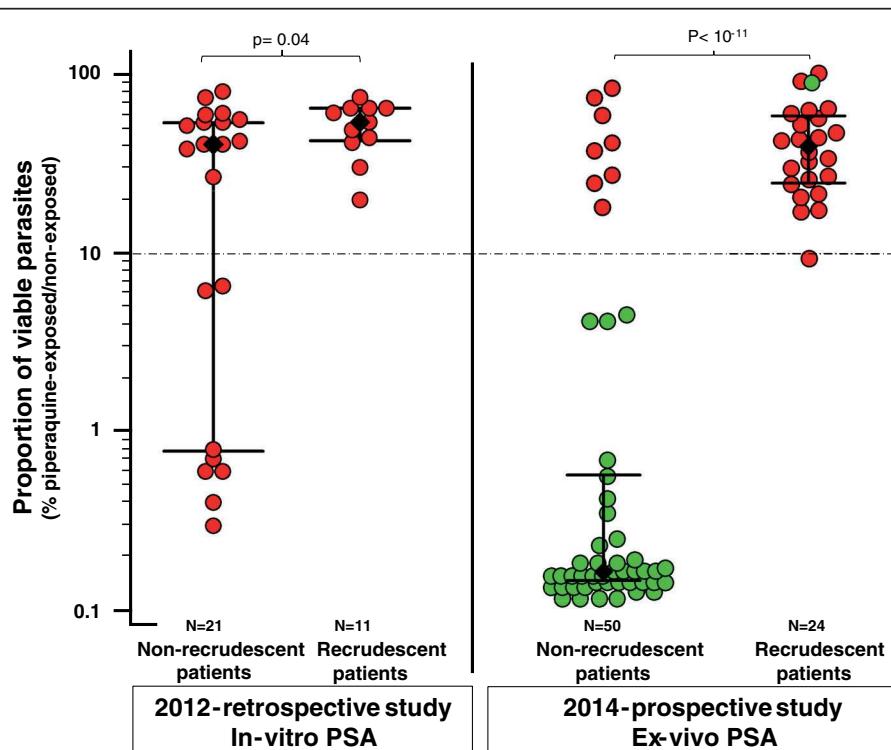


Fig. 2 Association between clinical dihydroartemisinin-piperaquine outcome and in vitro and ex vivo piperaquine survival assay (PSA) survival rates. In vitro and ex vivo PSAs were done with 0–3 h post-invasion rings from culture-adapted parasites isolated in 2012–2013 or parasites directly collected from patients with malaria in Rattanakiri, Siem Reap, Stung Treng, and Mondulkiri in 2014, respectively. Results from the in vitro and ex vivo PSAs are expressed as the proportion of viable parasites in the exposed or non-exposed cultures (Fig. 1). Isolates (collected on day 0) are dichotomized according to the clinical outcome of infection in patients enrolled and treated with a 3-day course of dihydroartemisinin-piperaquine (non-recrudescence or recrudescence of *P. falciparum* infections within 42 days, after PCR-correction). The median of the proportion of viable parasites was significantly higher in isolates from subsequently recrudescent than non-recrudescent patients (in vitro PSAs 51.9 % vs. 34.4 %, respectively, $P = 0.04$; ex vivo PSA: 39.2 % vs. 0.17 %, respectively, $P < 1 \times 10^{-11}$). Each circle represents a *P. falciparum* isolate. Red and green colors refer to K13 mutant alleles (C580Y or Y493H) and K13 wild-type alleles, respectively. The black diamonds, the horizontal lines and I bars represent the medians and interquartile ranges. The dotted grey line represents the 10 % survival rate cut-off that distinguishes piperaquine-resistant ($\geq 10\%$) from piperaquine-sensitive (<10 %) parasites in PSAs

eight candidate genes tested [13, 16, 23–26], specific mutations of *Pfcrt* and copy number variation of *Pfmdr1* were highly associated with piperaquine resistance (Additional file 5). The isolates containing parasites with a variant of the Dd2 *Pfcrt* allele carrying either 97Y, 343 L or 353 V had higher median survival rates compared to those harboring the Dd2 allele. *Pfmdr1* single copy parasites had higher median survival rates than *Pfmdr1* multi-copy parasites (48.1 % vs. 0.6 %, $P < 1 \times 10^{-3}$; Table 1).

Discussion

Declining efficacy of ACTs and, more specifically, of dihydroartemisinin-piperaquine, can jeopardize the gains obtained during the last decade in controlling malaria in Cambodia. The proportion of patients who experience late treatment failure with dihydroartemisinin-piperaquine is on the increase and, worryingly, this trend appears to be spreading eastwards in the country. Artemisinin resistance accounts for slower clearance rates in the first 3 days of treatment, but late recrudescence is believed to reflect incomplete efficacy of the long half-life partner drug. Although significant progress has been made recently in the detection of artemisinin-resistant *P. falciparum* parasites

[11, 17, 19], tools to detect piperaquine resistance more rapidly than the 42-day post-treatment outcome are urgently needed. The work reported here fills this important gap in providing a robust in vitro assay clearly differentiating resistant isolates from susceptible ones.

IC_{50} data from standard in vitro assays have proved so far to be inadequate to assess piperaquine resistance; IC_{50} values have not distinguished recrudescence and non-recrudescence isolates in patients treated with dihydroartemisinin-piperaquine [12, 13, 15, 16, 27]. The poor performance of the standard assays in this regard is possibly due to the relatively high frequency of non-interpretable curves observed frequently in assays of piperaquine resistant isolates. Indeed, most of the isolates collected from recrudescence patients (9/11) studied here gave non-interpretable curves (Additional file 3), although all had conventional response curves to the other drugs tested. These anomalous curves presented a paradoxical increase of [3 H]-hypoxanthine incorporation at piperaquine concentrations above 100–200 nM, the physiological concentration of piperaquine observed in blood in patients treated with a standard 3-day course of dihydroartemisinin-piperaquine during the first 3 days [21]. Several factors might contribute to such atypical

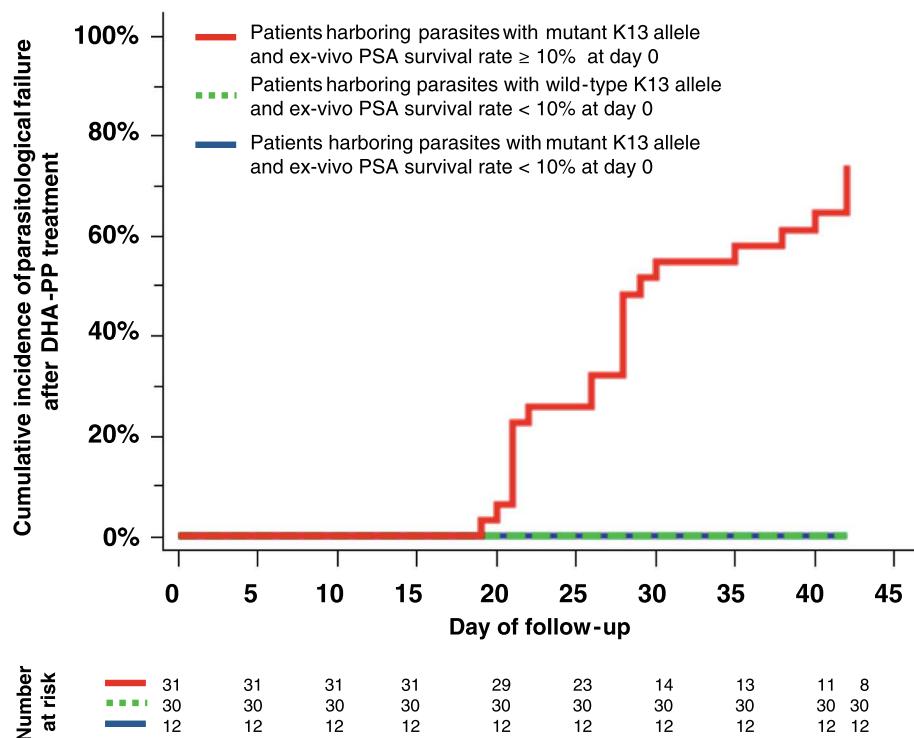


Fig. 3 Cumulative incidence of clinical failure within 42 days (after PCR-correction) in patients treated with a 3-day dihydroartemisinin-piperaquine course according to K13 allele (wild-type or mutant) and ex vivo piperaquine survival assay (PSA) survival rates of day 0 parasites. The cumulative incidence of clinical failure was significantly higher in patients infected on day 0 by isolates carrying a mutant K13 allele and presenting a PSA survival rate $\geq 10\%$ ($P < 1 \times 10^{-10}$, log rank test, Hazard Ratio = 14.3, 95 % CI, 4.6–44.6, Fig. 3). The survival proportion at day 42 for those patients was estimated 25.8 % (SD = 7.9 %)

profiles, reported previously for several ACT partner drugs, including altered transcriptional responses, increased protein production or nucleic acid precursor uptake for drugs ineffective on ring-stages, plate edge effects or mixed-clone infections [22]. This last factor can be excluded here as we used culture-adapted single clone lines as well as the insolubility of the piperaquine powder at high concentrations (>100 nM) in drug test wells, as presented in Additional file 6. Our data rather suggest that the paradoxical profiles might reflect an inducible mechanism of piperaquine resistance triggered at physiological concentrations (~200 nM). Inducible responses were avoided in the PSA by assessing the viability of the parasites over a 24 h period after the 48 h incubation. Importantly, non-interpretable curves were significantly more frequent for isolates with in vitro PSA survival rates $\geq 10\%$ (83 % vs. 0 %, $P < 1 \times 10^{-4}$; Additional file 1). This cannot be reliably used as a phenotype proxy of piperaquine resistance as the effect cannot be differentiated from failed assays. To overcome the limitations of the current assays and obtain a robust assessment of piperaquine resistance, we developed the PSA based on the detection of viable parasites after exposure to 200 nM for 48 h. The PSA was designed to mimic in vivo exposure of *P. falciparum* parasites to physiological concentrations of piperaquine (200 nM) for 48 h (all parasite stages – from 0–3 h early ring stages to 48 h schizonts – were then exposed to piperaquine, as the piperaquine half-life is ~9 days after a standard cure in treated patients, to globally assess the parasite susceptibility over its entire life cycle). The assay was not designed to investigate the piperaquine susceptibility to different parasite stages (by exposing the parasite stages to shorter pulses). These experiments deserve to be performed in future studies to comprehensively decipher the mechanism of action/resistance of *P. falciparum* to piperaquine. Despite these limitations, the PSA highlights, for the first time, that survival rates from the ex vivo testing strongly correlate with the clinical outcome of 3-day dihydroartemisinin-piperaquine treatment. All samples isolated at day 0 from recrudescent infections had a PSA survival rate $\geq 10\%$, a cut-off value that can be used to define resistance to piperaquine. The ex vivo PSA remarkably captured the survival capacity/potential of day 0 parasites exposed to piperaquine, which became overt in patients only several weeks later. This was probably facilitated by the low complexity of day 0 infections, which in the large majority consisted of single parasite clones.

In vitro PSA testings carried out with isolates harboring the C580Y K13 mutation showed that resistance to piperaquine is not directly related to K13 polymorphism as 8 of 21 C580Y, artemisinin-resistant parasites isolated from non-recrudescent infections were susceptible to

piperaquine (PSA <10 %; Fig. 2). However, the prospective study showed that essentially all parasites presenting a survival rate $\geq 10\%$ also carried a mutant K13 locus. This likely reflects the fact that, in patients, piperaquine resistance was selected from parasites that were already artemisinin-resistant, necessitating survival first to the fast acting drug (three short-pulses of dihydroartemisinin) and afterwards to the long-acting partner drug (piperaquine). The association with artemisinin resistance reflects this two-step selection process rather than being causal. In Western Cambodia, the extremely limited genetic diversity of the parasite populations means that almost all parasites carry K13 polymorphisms [17, 28, 29] and the selection for resistance to piperaquine was presumably correspondingly strong. Piperaquine resistance was also associated with other genetic polymorphisms. We confirm that single-copy *Pfmdr-1* (and consequently low mefloquine IC₅₀) is associated with piperaquine resistance. All piperaquine-resistant isolates (in vitro PSA survival rates $\geq 10\%$) had a single *Pfmdr-1* copy (median PSA survival rate = 48 % vs. 0.6 % in *Pfmdr-1* multiple copy isolates, $P = 0.0001$), but the reverse was not true, as isolates with a single copy of *Pfmdr-1* were not all piperaquine-resistant (Additional file 5). This finding strongly supports the recent recommendation of the Cambodian National malaria control programs for using artesunate plus mefloquine as first-line treatment in provinces where dihydroartemisinin-piperaquine failure rates are above 10 %. We also found a possible association with three independent *Pfcrt* mutations on the Dd2 genetic background (Y97, L343, and V353). Whether these associations reflect the structure of Cambodian parasite populations stemming from recent bottlenecks [28, 29] or a direct contribution to the phenotype remains to be investigated using genome-wide association studies and gene editing. The PSA will be particularly useful for such studies.

Conclusion

The data presented here demonstrate that the ex vivo PSA is a convenient method for monitoring piperaquine resistance in the field, especially in areas of artemisinin resistance such as Vietnam and Myanmar where dihydroartemisinin-piperaquine is the recommended first line treatment of uncomplicated falciparum malaria. Ex vivo PSA combined with K13 genotyping informing on both artemisinin and piperaquine resistance has the potential to provide timely evidence at the country level and complement the therapeutic efficacy studies to inform national malaria control programs and policymakers about existing or emerging risks of artemisinin and piperaquine resistance.

Consent to publish

A written informed consent was obtained from any enrolled patients for publication. A copy of the written consent is available for review by the Editor of this journal.

Additional files

Additional file 1: Patient information and corresponding data from in vitro piperaquine survival assays performed on 32 culture-adapted *P. falciparum* isolates from Cambodia collected on day 0 in 2012 from malaria patients treated with dihydroartemisinin-piperaquine. (PDF 94 kb)

Additional file 2: Patient information and corresponding data from ex vivo piperaquine survival assays performed on *P. falciparum* isolates from Rattanakiri, Siem Reap, Stung Treng and Mondulkiri provinces in 2014. (PDF 77 kb)

Additional file 3: Examples of interpretable piperaquine concentration-inhibition curves (Panel A) and non-interpretable piperaquine concentration-inhibition curves due to a paradoxical increase of apparent growth at high drug concentrations (Panel B: curves do not fit the data; Panel C: core criteria of curves are not acceptable). (PDF 40 kb)

Additional file 4: Flow chart of patients included in the final analysis of the 2014 retrospective study. Cambodia 2014 and isolates used in ex vivo assays. (PDF 52 kb)

Additional file 5: In vitro piperaquine survival assay survival rate distribution in 32 culture-adapted *P. falciparum* isolates from Cambodia in 2012 according to mutations or copy number variation in candidate genes significantly associated with piperaquine resistance (see Table 1). Panel A: mutations of *P. falciparum* chloroquine resistant transporter gene (*Pfcrt*); Panel B: copy number variation of *P. falciparum* multidrug resistance 1 gene (*Pfmdr-1*). (PDF 85 kb)

Additional file 6: Results of the piperaquine concentration measurements in culture supernatants of different wells in plate used for the classical isotopic assay, containing increasing piperaquine concentrations (from 1.91 nM in PPQ11 to 2000 nM in PPQ1). (DOCX 12 kb)

Abbreviations

ACT: Artemisinin combination therapy; IC: Inhibitory concentration; PSA: Piperaquine survival assay.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

VD, PJG, OMP, BW, and DM contributed to study design. NK, SC, RE, CK, KL, MK, genotyped parasites. AD, NKL, SKE, VD, and BW performed in vitro and ex vivo drug assays. RL, DL, and RH gathered clinical data. JB and FA performed analysis of whole genome sequencing data. VD, BW, and DM analyzed data. VD, PJG, BW, OM-P, and DM wrote the first manuscript.

All authors have read and approved the final manuscript.

Acknowledgments

We thank all patients enrolled in the therapeutic efficacy studies, the health centres staff and the staff from the National Center for Parasitology, Entomology and Malaria Control in Cambodia for their support. We are grateful to Joel Tarning (Mahidol Oxford Tropical Medicine Research Unit, Bangkok, Thailand; Centre for Tropical Medicine, University of Oxford, Oxford, United Kingdom) who performed the piperaquine concentration measurements. This work was funded by grants from Institut Pasteur du Cambodge, USAID and BMGF through the World Health Organization, and Institut Pasteur Paris. PJG is member of the Worldwide Antimalarial Resistance Network supported by the Bill and Melinda Gates Foundation. VD

is supported by a doctoral fellowship from the International Division, Institut Pasteur. None of the funders had any role in study design, data collection, analysis, and interpretation, writing of the manuscript and in the decision to submit the manuscript for publication.

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Received: 30 August 2015 Accepted: 30 November 2015

Published online: 22 December 2015

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Chapitre VI. Découverte d'un marqueur moléculaire candidat pour la résistance à la PPQ chez *P. falciparum*

1. Introduction

L'augmentation spectaculaire des taux d'échecs à la DHA-PPQ au Cambodge, que l'on estime aujourd'hui atteindre 60% dans certaines régions, met l'accent sur la nécessité de développer urgentement des outils de détection et de surveillance rapides et efficaces. Un pas en avant conséquent a été fait avec la récente mise au point du PSA, le nouveau test phénotypique présenté dans l'article précédent⁷¹, qui s'est montré capable de détecter la résistance à la PPQ et les échecs de traitement plus efficacement que les tests classiques d'IC₅₀. Ce test représente donc un outil intéressant pour la recherche de signatures moléculaires associées à la résistance à la PPQ.

Nous avons utilisé les informations phénotypiques fournies par le PSA pour comparer les séquences des exomes de différents isolats cambodgiens de *Plasmodium falciparum*, ayant été classés comme susceptibles ou résistants à la PPQ selon leur taux de survie en PSA *in vitro*. Les exomes de ces lignées parasitaires ont été analysés afin de mettre en évidence des polymorphismes nucléotidiques (*single nucleotide polymorphisms*, SNPs) et des variabilités de nombres de copies (*copy number variations*, CNVs).

2. Résultats et discussion

Nous avons obtenu les séquences des exomes de 31 isolats résistants à l'ART (porteurs de la mutation K13 C580Y), collectés en 2012 puis adaptés en culture, dont 23 résistants à la PPQ (taux de survie en PSA *in vitro* $\geq 10\%$) et 8 susceptibles à la PPQ (taux de survie en PSA *in vitro* $< 10\%$). Nous avons détecté de forts signaux d'amplification chez le groupe résistant à la PPQ dans une région du chromosome 14 codant les plasmepsines (*PfPMS*), des protéases

impliquées dans la digestion de l'hémoglobine. Le nombre de copies des gènes *PfPM2* et *PfPM3* était significativement plus élevé chez les lignées résistantes à la PPQ ($r=0.83$ [95% Confidence Interval (CI) 0.67-0.91], $P<0.0001$ and $r=0.85$ [95% CI 0.71-0.93], $P<0.0001$). À l'inverse, un ensemble de gènes sur le chromosome 5 comprenant *Pfmdr1* présentait une amplification chez les lignées susceptibles à la PPQ ($P=0.015$, Wilcoxon test).

Pour confirmer cette association, nous nous sommes concentrés sur le gène *PfPM2*, situé au centre de la région amplifiée chez les souches résistantes à la PPQ et présentant l'association statistique la plus forte. Le développement d'une méthode de qPCR adaptée nous a permis de montrer que le nombre de copies de *PfPM2* était corrélé aux taux de survie en PSA *ex vivo* de 134 isolats cambodgiens : la médiane des taux de survie en PSA était significativement plus élevée pour les isolats porteurs d'au moins 2 copies de *PfPM2* (51.7% contre 0.004% pour les isolats sans amplification de *PfPM2*, $P<0.0001$, Mann-Whitney test).

Nous avons par la suite exploré l'association entre le nombre de copies de *PfPM2* et l'issue thérapeutique après un traitement standard de DHA-PPQ sur 725 échantillons sanguins collectés chez des patients avant administration du traitement et suivis durant 42 jours : une amplification du gène *PfPM2* était associée à un rapport de risque d'échec de traitement de 20.4 (95% CI 9.1-45.5, $P<0.001$), et prédisait les échecs de traitement à la DHA-PPQ avec une sensibilité de 0.94 (95% CI 0.88-0.98) et une spécificité de 0.77 (95% CI 0.74-0.81).

Enfin, nous nous sommes intéressés au lien entre les tendances spatio-temporelles d'efficacité de la DHA-PPQ au Cambodge et l'évolution du nombre de copies de *PfPM2* des parasites cambodgiens. Nous avons analysé des échantillons collectés à l'ouest et à l'est du Cambodge entre 2002 et 2015. Dans les provinces de l'ouest, la proportion de parasites porteurs d'une amplification de *PfPM2* a augmenté de 27.9% en 2008-2009 à 91.2% en 2014-2015. Dans les provinces de l'est, les parasites porteurs d'une amplification de *PfPM2* étaient peu

fréquents jusqu'en 2012-2013 (3.2%), mais ont atteint une proportion de 45.5% en 2014-2015. Ces données corrèlent bien avec l'augmentation des taux d'échecs cliniques à la DHA-PPQ dans ces deux régions. Nous avons également montré par un modèle de régression linéaire que l'efficacité clinique de la DHA-PPQ à J42 chutait sous le seuil des 90% lorsque la proportion de parasites porteurs d'une amplification *PfPM2* et d'un gène *K13* muté dépassait 22%.

Nos travaux ont permis de montrer que la résistance à la PPQ au Cambodge était associée à une amplification des gènes *PfPM2* et *PfPM3*, qui encodent des protéases impliquées dans la digestion de l'hémoglobine. L'amplification du gène *PfPM2* constitue un marqueur moléculaire candidat pour la détection et la surveillance de la résistance à la PPQ dans les zones endémiques. Combiné à l'analyse du polymorphisme de *K13* et du nombre de copies de *Pfmdr1*, ce nouveau marqueur moléculaire apporte des informations critiques pour les politiques de traitements du paludisme et les mesures d'endiguement. Nous notons toutefois que l'association entre la résistance à la PPQ et l'amplification de *PfPM2-3* n'établit pas une preuve de causalité : les découvertes présentées ici devront être complétées par l'étude des conséquences cellulaires de cette amplification sur la réponse du parasite à la PPQ, sa fitness et sa transmissibilité.

3. Article 6

A surrogate marker of piperaquine-resistant Plasmodium falciparum malaria

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The Lancet Infectious Diseases, accepté.

1 **A surrogate marker of piperaquine-resistant *Plasmodium falciparum* malaria**

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3 Running title: A molecular marker of piperaquine-resistant malaria
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84 **Abstract: (299 words); Main text (3463 words – without panel); Figures (4); Table (2)**

85 **Summary**

86 **Background.** Western Cambodia, the epicentre of *Plasmodium falciparum* multidrug resistance,
87 is currently facing high rates of dihydroartemisinin-piperaquine (DHA-PPQ) treatment failures,
88 indicating resistance to both artemisinin derivatives and piperaquine. Genetic tools to detect
89 these multidrug-resistant parasites are needed. Artemisinin resistance can be tracked using the
90 K13 molecular marker, but no marker exists for piperaquine resistance.

91 **Methods.** We obtained blood samples from *P. falciparum*-infected Cambodian patients treated
92 with DHA-PPQ and followed-up for 42 days during the years 2009-2015 and established in-vitro
93 and ex-vivo susceptibility profiles for a subset using piperaquine survival assays (PSA). We
94 determined whole-genome sequences by Illumina paired-reads sequencing, copy number
95 variations by qPCR, RNA levels by qRT-PCR, and protein levels by immunoblotting.

96 **Findings.** Whole-genome exon sequence analysis of 31 culture-adapted parasite lines
97 associated amplification of the *plasmepsin* 2-3 cluster (*PfPM2* and *PfPM3*) with in-vitro
98 piperaquine resistance. Ex-vivo PSA profiles of 134 isolates correlated with *PfPM2* gene copy
99 number. In 725 patients treated with DHA-PPQ, multicopy *PfPM2* in the sample collected
100 before treatment was associated with an adjusted hazard ratio for treatment failure of 20.4
101 (95% CI 9.1–45.5, p<0.0010). Multicopy *PfPM2* predicted DHA-PPQ failures with 0.94 (95% CI
102 0.88–0.98) sensitivity and 0.77 (95% CI 0.74–0.81) specificity. Analysis of samples collected
103 across the country from 2002 to 2015 showed that the geographic and temporal increase of the
104 proportion of multicopy *PfPM2* parasites was highly correlated with increasing DHA-PPQ
105 treatment failure rates. DHA-PPQ efficacy at day 42 fell below 90% when the proportion of
106 multicopy *PfPM2* parasites exceeded 22%.

107 **Interpretation.** Piperaquine resistance in Cambodia is strongly associated with amplification of
108 *PfPM2-3*, encoding hemoglobin-digesting proteases, regardless the location. Multicopy *PfPM2*
109 constitutes a surrogate molecular marker to track piperaquine resistance. A molecular toolkit
110 combining *PfPM2* with *K13* and *Pfmdr1* monitoring should provide timely information for
111 antimarial treatment and containment policies.

112 **Funding.** Institut Pasteur in Cambodia, Institut Pasteur Paris, National Institutes of Health,
113 World Health Organization, Agence Nationale de la Recherche, Investissement d'Avenir
114 program, Laboratoire d'Excellence "Integrative Biology of Emerging Infectious Diseases".

115

116 **Keywords** *Plasmodium falciparum*, malaria, dihydroartemisinin, piperaquine, *Plasmepsins*,
117 *Pfplasmepsin2*, copy number variation, *Pfmdr1*, K13 polymorphisms, multidrug resistance,
118 Cambodia, elimination

119

120

121 **Introduction**

122 Antimalarial efficacy of artemisinin-based combination therapies (ACTs), the first-line treatment
123 for uncomplicated *Plasmodium falciparum* malaria, relies on both fast-acting artemisinin (ART)
124 derivatives and long-lasting partner drugs. Resistance to ARTs, which is now fixed in western
125 Cambodia and observed across Southeast Asia, increases the proportion of parasites surviving a
126 3-day course of an ACT. Resistance to the partner drug is a greater risk when more parasites
127 survive ARTs. The reduced efficacy of ARTs and partner drugs translates into late treatment
128 failures and prolonged parasite carriage, thereby increasing the transmission potential of drug-
129 resistant infections.

130

131 In Cambodia, the artesunate-mefloquine ACT was chosen as the first-line drug in 2001. By 2008,
132 the high frequency of treatment failures in western provinces, the epicenter of *P. falciparum*
133 multidrug resistance, led to its replacement by dihydroartemisinin-piperaquine (DHA-PPQ) in
134 2008, and later throughout Cambodia in 2010. In recent years the spread of ART-resistant *P.*
135 *falciparum*, from western Cambodia to neighboring provinces,¹⁻⁵ has been followed by a
136 spectacular increase in DHA-PPQ failure rates. Failures are currently estimated to reach 60%,⁶⁻¹⁰
137 indicating a dramatic expansion of piperaquine (PPQ) resistance. Until now, the detection of
138 PPQ resistance has been based on logically demanding 42-day follow up studies of DHA-PPQ
139 treated patients.¹¹ The recently developed in-vitro Piperaquine Survival Assay (PSA)⁷ has been
140 shown with in-vitro culture-adapted parasites and freshly collected ex-vivo patient isolates to
141 detect PPQ-resistance and treatment failure more reliably than classic dose-response assays.⁷
142 The in-vitro PSA therefore provides a reliable tool to identify molecular signatures associated
143 with resistance.

144

145 Here, we used the PSA-based phenotypic information to identify genetic marker(s) of PPQ
146 resistance and study their association with DHA-PPQ treatment failures. First, the exomes of
147 culture-adapted ART-resistant Cambodian *P. falciparum* lines dichotomized as PPQ-susceptible
148 or -resistant based on their PSA survival rates⁷ were compared for single nucleotide

149 polymorphisms (SNPs) and copy number variations (CNVs). This identified an increased copy
150 number of the *Pfplasmepsin2/Pfplasmepsin3* cluster as a putative genetic signature associated
151 with in-vitro PPQ resistance. Increased *Pfplasmepsin2* (*PfPM2*) gene copy number was then
152 assessed as a candidate resistance marker in 134 isolates with documented ex-vivo PSA survival
153 rates and in 725 blood samples collected during the years 2009-2015 from Cambodian patients
154 treated with DHA-PPQ and followed up for 42 days. Finally, we investigated the geographic and
155 temporal distribution of multicopy *PfPM2* parasites in the country from 2002 to 2015 and its
156 correlation with DHA-PPQ treatment failures. Our data show that amplification of the *PfPM2*
157 locus is a surrogate marker of PPQ resistance, which can be used to predict the risk of DHA-PPQ
158 treatment failure in ART-resistant areas. We also provide insights into the possible role of the
159 digestive vacuole in PPQ resistance and the stepwise selection process that has resulted in
160 multidrug resistance in Cambodian *P. falciparum* parasites.

161

162 **Materials and methods**

163 **Study sites and patients.**

164 Patients with *P. falciparum* malaria were enrolled in clinical studies conducted at health centers
165 located across Cambodia during the years 2009-2015 (Table_1, Appendix_1). After obtaining
166 written informed consent, patients were treated with DHA-PPQ (Duo-Cotecxin®,
167 dihydroartemisinin 40mg and piperaquine 320mg, Zhejiang Holley Nanhu Pharmaceutical Co.
168 Ltd, China) and followed up for 42 days as previously described.^{7,8,10} The endpoint to evaluate
169 DHA-PPQ efficacy was the proportion of PCR-corrected recrudescent *P. falciparum* infections at
170 day 42.¹¹ All studies were approved by the Ethical Committee for Health Research of the
171 Cambodian Ministry of Health. Clinical trials were registered at the Australian New Zealand
172 Clinical Trials Registry (ACTRN 12615000793516, 12612000184875, 12612000183886,
173 12612000181808 and 12614000344695).

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175

176

177 **Sample collection**

178 Blood samples were collected into acid-citrate-dextrose tubes (Becton-Dickinson, USA) before
179 treatment and sent to Institut Pasteur in Cambodia within 24 hours. A sub-set of freshly
180 collected samples was used to perform the ex-vivo PSA.⁷ All samples were cryopreserved in
181 glycerolyte. Red cell pellets were stored at -20°C for molecular studies. Blood spots were
182 prepared on day 0 and when applicable on the day of recrudescence.

183

184 **Laboratory investigations**

185 Cryopreserved parasites were culture-adapted as described.¹² Susceptibility to PPQ was
186 investigated using in-vitro PSA for culture-adapted parasites and ex-vivo PSA for fresh isolates.
187 Survival rates were assessed microscopically and parasites with a survival rate ≥10% were
188 considered PPQ-resistant.⁷ *msp1*, *msp2* and *glurp* polymorphisms were determined to
189 distinguish recrudescent from new infections.¹³ Sequencing of the K13-propeller domain was
190 used to screen for ART-resistance.¹ Whole-genome sequencing was performed using Illumina
191 paired-reads sequencing.¹ Data were integrated into the Whole-genome Data Manager (WDM)
192 database¹⁴ and exomes of PPQ-resistant and -sensitive lines were compared after excluding
193 low-coverage positions (i.e., lower than 25% of the genome-wide mean coverage). Genes from
194 highly variable multigene families (*var*, *rifin*, *phist* and *stevor*) were excluded.¹ CNVs and SNPs
195 were investigated using PlasmoCNVScan and Phen2gen software's (see Appendix_2 for
196 details).¹⁴ *PfPM2* and *Pfmdr1* copy number was determined by qPCR (Appendix_3). Steady-
197 state *PfPM2* mRNA levels were measured by RT-qPCR (Appendix_4) and *PfPM2* protein
198 expression by immunoblotting (Appendix_2).

199

200 **Statistical analysis**

201 Data were analyzed with MedCalc version 12 (Mariakerke, Belgium). Kruskal-Wallis or Mann-
202 Whitney tests were used for non-parametric comparisons and the Student's t-test or one-way
203 analysis of variance were used for parametric comparisons. For proportions (expressed with
204 percentages and 95% confidence intervals), chi-squared or Fisher's exact tests were performed.

205 Manhattan plots were generated using the SNPEVG software.¹⁵. We conducted SNP-wise
206 analysis and used the exact Fisher's exact test to identify significant SNPs differences between
207 PPQ-resistant and -sensitive parasite lines. We tested CNV for differential distribution between
208 piperaquine-resistant and -sensitive parasite lines using a non-parametric Wilcoxon rank-sum
209 test. The Bonferroni and the Benjamini-Hochberg corrections were used to evaluate genome-
210 wide significance and adjust p-values when statistical tests were performed simultaneously on
211 a single data set (Appendix_2). Relative risks were estimated using the Mantel-Haenszel test.
212 Relationships between a cumulative risk of failure at day 42 and molecular signatures
213 associated with PPQ resistance were assessed by survival analysis. Curves were compared with
214 the Mantel-Haenszel log-rank test. The Cox proportional-hazards regression model was used to
215 evaluate association between parasite genotypes (*K13* mutations, *PfPM2* and *Pfmdr1* copy
216 number), sampling locations and treatment responses. A linear regression analysis was used to
217 evaluate the relationship between DHA-PPQ efficacy and the proportion of parasites with
218 multicopy *PfPM2*. We deemed p-values of less than 0.05 as significant.

219

220 **Role of the funding source**

221 The sponsors of this study had no role in study design, data collection, data analysis, data
222 interpretation, writing of the report and the decision to submit. The corresponding author had
223 full access to all data in the study and final responsibility for the decision to submit for
224 publication.

225

226 **Results**

227 During 2009-2015, 725 patients were enrolled in clinical studies to assess the efficacy of the
228 standard 3-day DHA-PPQ treatment. By 2015, the cumulative proportion of *P. falciparum*
229 recrudescence at day 42 after PCR correction was 16.4% (119/725), ranging from 0 to 62.5%
230 depending on the site and the year of study (Table_1, Figure_1).

231

232

233 **Molecular signatures associated with PPQ-resistance.**

234 Whole-genome sequences were obtained from 31 ART-resistant (*K13 C580Y* mutant) culture-
235 adapted parasite lines collected in western Cambodia in 2012, including 23 PPQ-resistant and
236 8 PPQ-sensitive lines as defined by their in-vitro PSA survival rates (Table_2). We observed a
237 total of 120,691 exomic (coding sequence) SNPs. Genome-wide association analyses of SNPs
238 identified significant differences between resistant and sensitive lines at two positions located
239 in adjacent genes on chromosome 4: position 896588 of PF3D7_0420000 (encoding a putative
240 zinc finger protein) ($p<3.56\times 10^{-7}$, Fisher's exact test; $p=0.042$ after Bonferroni correction) and
241 position 908385 of PF3D7_0420100 (encoding a Rio2 serine/threonine protein kinase)
242 ($p<3.56\times 10^{-7}$, Fisher's exact test; $p=0.042$ after Bonferroni correction). However, these
243 positions (and indeed the sequences of both genes) were ambiguous with variable proportions
244 of wild type and mutant nucleotides, precluding identification of specific resistance-associated
245 mutations, and were not studied further (Appendix_5).

246

247 In contrast, signals of gene amplification were detected in the PPQ-resistant group for two
248 adjacent genes from the cluster located on chromosome 14 that encode hemoglobin-digesting
249 proteases known as plasmepsins (*PfPMs*) ($p=0.03795$ Wilcoxon test with Benjamini-Hochberg
250 correction, Figure_2). Irrespective of piperaquine susceptibility, all *PfPM3* sequences were wild-
251 type and all *PfPM2* sequences showed a Q442H *PfPM2* polymorphism, which has been
252 frequently observed in reference laboratory lines or wild isolates
253 (<http://plasmodb.org/plasmo>). The correlation between in-vitro PSA survival rates and
254 *PfPM2/PfPM3* copy number was highly significant ($r=0.83$ [95% Confidence Interval (CI) 0.67-
255 0.91], $p<0.0001$ and $r=0.85$ [95% CI 0.71-0.93], $p<0.0001$). We observed three different profiles
256 of DNA expansion (Appendix_6, Table_2). In-vitro PSA survival rates were significantly lower in
257 parasites harboring DNA expansion type 2 (N=6, PSA median value=34.1%, IQR=25.8-40.0%)
258 compared to those harboring DNA expansion type 1 (N=13, PSA median value=51.8%,
259 IQR=45.6-61.7%, $p=0.006$, Mann-Whitney test) or type 3 (N=3, PSA median value=58.7%,
260 $p=0.02$, Mann-Whitney test).

261 Conversely, a cluster of 5 genes on chromosome 5 (PF3D7_0531700, PF3D7_0522900,
262 PF3D7_0523000, PF3D7_0523100, PF3D7_0523200), which included *Pfmdr1*, presented
263 increased copy numbers in sensitive lines. *Pfmdr1* was amplified in 5 of 8 PPQ-sensitive lines
264 but 0 of 23 PPQ-resistant lines ($p=0.015$, Wilcoxon test) (Appendix_7).

265

266 ***PfPM2* CNV and ex-vivo PPQ susceptibility**

267 To confirm the association between *PfPM* CNV (copy number variation) and ex-vivo PSA survival
268 rate, we used *PfPM2* as an amplicon reporter. First, we optimized a qPCR method to assess
269 *PfPM2* gene copy number (Appendix_3). *PfPM2* copy number detected by qPCR was 100%
270 concordant with the whole-genome sequencing estimates for the 31 culture-adapted parasites
271 ($p<0.0001$, Fisher test). From a set of 134 isolates with known ex-vivo PSA profiles, *PfPM2* was
272 amplified in 67 of 69 PPQ-resistant parasites (50, 15 and 2 isolates with 2, 3 or 4 *PfPM2* copies,
273 respectively), and 0 of 65 PPQ-susceptible parasites (Figure_3). The median ex-vivo PSA survival
274 rate was significantly higher in isolates with ≥ 2 *PfPM2* copies compared to those with
275 unamplified *PfPM2* (51.7% [IQR 29.7-75.1%] vs. 0.004% [IQR 0.003-0.39%], $p<0.0001$, Mann-
276 Whitney test). An increased *PfPM2* copy number predicted ex-vivo PPQ resistance with a
277 sensitivity of 0.97 (95%CI 0.90-0.99) and specificity of 1.0 (95%CI 0.65-1). K13 polymorphisms
278 were detected in 65 PPQ-resistant and 17 PPQ-susceptible isolates (Figure_3). Only 4 of 69
279 PPQ-resistant isolates harbored a wild-type K13 sequence. In a multiple regression analysis,
280 increased *PfPM2* copy number was more strongly associated than K13 mutations with in-vitro
281 PPQ resistance ($r_{\text{partial}}=0.94$, $p<0.0001$ and $r_{\text{partial}}=0.25$, $p=0.004$, respectively).

282

283 **mRNA and protein levels in single and multicopy *PfPM2* parasites**

284 *PfPM2* transcript levels were 4.1- to 5.3-fold higher in the PPQ-resistant line ID_6320 as
285 compared to the PPQ-sensitive line ID_6267 at all time points of the intra-erythrocytic cycle
286 investigated. *PfPM2* protein levels were at least two-fold higher in PPQ-resistant parasites

287 ID_6408 compared to the sensitive line ID_6267 (Appendix_8). This is consistent with increased
288 product levels in the multicopy *PfPM2* lines studied. However, further work is required to
289 expand this analysis to additional lines.

290

291 ***PfPM2* CNV and DHA-PPQ treatment outcome**

292 We next explored the relationship between *PfPM2* CNV and DHA-PPQ treatment outcome in
293 the isolates from 725 patients collected before DHA-PPQ treatment, of whom 119 experienced
294 a recrudescence between day 12 and day 42 (Figure_1). *PfPM2* was unamplified, 2-copy or \geq 3-
295 copy in 476/725 (65.7%), 153/725 (21.1%) or 96/725 (13.2%) isolates, respectively. Only 7 of
296 476 patients (1.5%) with unamplified *PfPM2* parasites had recrudesced by day 42 compared
297 with 112/249 (45.0%) patients infected with multicopy *PfPM2* parasites (RR=22.8 [95%CI: 10.7–
298 48.6]; p<0·0001). Recrudescence was more frequent for isolates with \geq 3 compared with 2
299 *PfPM2* copies (52/96, 54.2% vs. 60/153, 39.2%, p=0.02). The cumulative incidence of DHA-PPQ
300 treatment failure increased with increasing *PfPM2* gene copies: unamplified vs. 2 copies
301 (HR=32.2 [95%CI: 17.9–58.0], p<0·0001), unamplified vs. 3 copies (HR=49.0 [95%CI: 23.0–
302 104.2], p<0·0001), or 2 copies vs. \geq 3 copies (HR=1.53 [95%CI: 1.04–2.25], p=0.02) (Figure_4A).
303 The mean time to recrudescence decreased with increasing *PfPM2* copy number: 41.9 days
304 (95%CI: 41.8–42.0) for patients with unamplified *PfPM2*, 36.0 days (95%CI: 34.6–37.4) for those
305 with 2 copies, or 34.0 days (95%CI: 32.1–35.9) for those with \geq 3 copies. An increased *PfPM2*
306 copy number predicted DHA-PPQ treatment failures with a sensitivity of 0.94 (95%CI: 0·88–
307 0·98) and a specificity of 0.77 (95%CI: 0·74–0·81). The AUC (area under the ROC curve) was 0.86
308 (95% CI 0.83–0.88), significantly different from 0.5 (p<0.0001).

309

310 **K13 propeller polymorphism and *Pfmdr1* gene amplification**

311 Among the 725 patients treated with DHA-PPQ, K13 mutants were detected in 443/725 (61.1%)
312 day 0 isolates (Figure_4B). Of these, 116/443 (26.2%) were from patients who failed DHA-PPQ
313 treatment by day 42 compared to 3 of 282 (1.1%) from patients harboring K13 wild-type
314 parasites (RR=24.6 [95%CI: 7.9–76.7], p<0.0001). A single *Pfmdr1* gene copy was detected in

315 610/725 (84.1%) day 0 isolates. DHA-PPQ failures were observed in 112/610 patients (18.4%)
316 infected with parasites harboring a single *Pfmdr1* copy and 7/115 (6.1%) of patients infected
317 with multicity *Pfmdr1* parasites (RR=3.0 [95%CI: 1.4–6.3]; p=0.003). The cumulative incidence
318 of DHA-PPQ treatment failure did not increase with increasing age (stratified in 3 classes: 0-15,
319 16-30 and >30 years, p=0.1809, Logrank test) or with increasing parasite numbers in isolates
320 collected before DHA-PPQ treatment (stratified in 4 classes: <5000, 5001-20000, 20001-50000
321 and >50000 parasites per µl, p=0.4612, Logrank test).

322
323 After controlling for *K13* and *Pfmdr1* genotypes in a Cox proportional-hazards regression model,
324 *PfPM2* copy number (any increase compared to non-amplification) was the most significant
325 molecular signature associated with DHA-PPQ treatment failure (AHR=20.4 [95%CI: 9.1–45.5],
326 p<0.0010), followed by *K13* mutation (AHR=5.5 [95%CI: 1.7-18.3], p=0.005) then *Pfmdr1* single
327 copy (AHR=2.05 [95%CI: 0.95-4.42], p=0.06). The cumulative incidence of DHA-PPQ treatment
328 failure among patients harboring ART-resistant parasites (i.e. an artemisinin resistance-
329 associated *K13* mutation) increased significantly with *PfPM2* copy number: unamplified (3.3%,
330 7/208) vs. ≥2 copies (46.4% 109/235; HR=17.5 [95%CI: 12.2-25.2]).

331
332 **Geographic and temporal distribution of PfM2 and DHA-PPQ efficacy in Cambodia**
333 *PfPM2* CNVs were investigated in 1252 samples collected across Cambodia from 2002 to 2015
334 (i.e., before and after the introduction of DHA-PPQ). This included 527 archived isolates in
335 addition to the 725 studied above (Appendix_9). A longitudinal sampling was performed in
336 Pailin (western Cambodia) and Rattanakiri (eastern Cambodia), where PPQ-DHA was introduced
337 in 2008 and 2010, respectively. In Pailin, the proportion of multicity *PfPM2* parasites increased
338 from 27.9% (19/68) in 2008-2009 to 91.2% (52/57) in 2014-2015. In Rattanakiri, multicity
339 *PfPM2* parasites were infrequent until 2012-13 (3.2%, 1/31) but increased to 45.5% (40/88) in
340 2014-2015 (Appendix_9, Panel_A). A steady increase of multicity *PfPM2* parasites after
341 introduction of DHA_PPQ was observed in other provinces as well (Preah Vihear, Pursat)
342 (Appendix_9, Panel_B).

343 In the 12 sites where DHA-PPQ efficacy studies were conducted in 2009-2015, the proportion of
344 multicopy *PfPM2* isolates was negatively correlated with day 42 cure rates ($r=0.89$ [95%CI: 0.77-
345 0.95]; $p<0.0001$) (Appendix_10, Panel_A). A Cox regression model showed that the risk to be
346 recrudescent following a DHA-PPQ 3-day course was significantly associated ($p<2.10^{-16}$) with
347 the presence of multicopy *PfPM2* parasites on day 0 regardless of the site of enrollment
348 (Appendix_10, Panel_B). A linear regression model showed that DHA-PPQ clinical efficacy at day
349 42 fell below 90% when the proportion of multicopy *PfPM2* parasites on K13-mutant genetic
350 background rose above 22%.

351

352 **Discussion**

353 Following reports of increasing failure of artesunate-mefloquine in western Cambodia, DHA-
354 PPQ was adopted in 2008 in the western provinces and implemented nationwide in 2010.
355 Resistance to this combination has recently accelerated to levels that render it widely
356 ineffective.¹ The dearth of alternatives creates a perilous situation whereby these multidrug-
357 resistant infections might become untreatable and spread to other malaria-endemic regions.

358

359 The strategy used here to search for genetic associations with PPQ resistance relied on
360 genome-wide sequence comparisons of a set of ART-resistant parasite lines collected in
361 western Cambodia in 2012, all harboring the C580Y artemisinin resistance mutation and
362 presenting in-vitro PSA survival rates indicative of PPQ resistance or susceptibility. We reasoned
363 that such a focused sampling in a geographically restricted population would reduce the genetic
364 "noise" of artemisinin responses and population structure. Results identified amplification of
365 the *PfPM2-3* cluster as a putative genetic event associated with PPQ resistance. To confirm this
366 association across the country, we focused on *PfPM2*, located in the center of the amplicon.
367 *PfPM2* amplification strongly correlated with ex-vivo PSA survival rates irrespective of ART
368 susceptibility and was highly predictive of DHA-PPQ failures in all geographic areas of
369 Cambodia. *PfPM2* amplification thus represents an informative marker for PPQ resistance.

370

371 The strong association between K13 polymorphisms and *PfPM2* amplification in the Cambodian
372 parasites studied herein most likely reflects the history of drug selection in Cambodia. The
373 proportion of isolates with different *K13-PfPM2* combinations (Appendix_11) is consistent with
374 a stepwise selection first for ART-resistance then for PPQ-resistance. This is in line with the
375 delayed appearance of multicopy *PfPM2* parasites in eastern provinces where the emergence
376 of ART resistance was delayed compared to western provinces (Appendix-9). DHA-PPQ failures
377 were rare in eastern Cambodia by 2013, confirming observations by others⁶ but increased
378 steadily in 2014 to reach high frequency by 2015. Most (94.1%, 112/119) DHA-PPQ failures had
379 a single gene copy of *Pfmdr1*, confirming earlier reports of DHA-PPQ failure cases.⁶⁻⁹ The
380 presence of single copy *Pfmdr1* is consistent with data reported for in vitro-selected PPQ-
381 resistant Dd2 parasites¹⁶ and analysis of field samples from Cambodia, suggesting opposing
382 resistance mechanisms against these molecules.¹⁷ We did not observe the *Pfcrt* C101F mutation
383 observed in a PPQ-pressured parasite line selected in-vitro. Thus, our data show that although
384 the most informative marker for PPQ-resistance is *PfPM2* copy number, mutation of K13
385 alongside a single *Pfmdr1* gene copy contribute to the DHA-PPQ failure phenotype. This does
386 not exclude that additional genes contribute to the PPQ-resistance. In particular, the
387 significance of the mutations observed for PF3D7_0420000 and PF3D7_0420100 is unclear.
388 Whether the notable sequence heterogeneity of both genes reflected ongoing purifying
389 selection associated with PPQ resistance or loss of mefloquine resistance is uncertain. Analysis
390 of a larger number of isolates with documented phenotypes for both mefloquine and PPQ is
391 needed to address this question.

392

393 Drug-selected gene amplification is a well-known phenomenon in malaria parasites.¹⁸⁻²¹ The
394 size of the amplicons on chromosome 14 varied depending on the isolate, as reported for
395 *Pfmdr1*.²² Gene amplification, which is more frequent than point mutation in *P. falciparum*
396 parasites²² is consistent with the remarkably rapid rise and spread of PPQ-resistance in
397 Cambodia. Conversely, *Pfmdr1* de-amplification, consistent with regained susceptibility to
398 mefloquine, occurred in Cambodia in the recent years,^{6,8,23} and, as shown here, is associated

399 with the emergence of PPQ-resistant strains.

400

401 Plasmepsins are expressed during the intra-erythrocytic asexual blood stage cycle as well as by
402 sexual stage gametocytes that can be transmitted to the mosquito vector. All four plasmepsins
403 (*PfPM1-4*) are located in the digestive vacuole of intra-erythrocytic developmental forms where
404 they engage in different steps of hemoglobin degradation. Studies of parasites disrupted in the
405 *PfPM* genes pointed to redundancy in the hemoglobin degradation machinery.²⁴ To our
406 knowledge, there are no reported studies about the consequences of overexpressing these
407 proteases. We show here that *PfPM2* amplification is associated with a notable increase of
408 steady state mRNA and protein levels in two culture-adapted isolates. This observation needs
409 to be confirmed with additional isolates. A reasonable hypothesis is that the amplification of
410 plasmepsins overcomes the inhibitory effect of PPQ on hemoglobin degradation and heme
411 detoxification, possibly by reducing levels of reactive heme species that are preferred
412 substrates for PPQ binding. PPQ-treated trophozoites have been shown to possess large
413 digestive vacuoles containing membrane-bound packets of undigested hemoglobin.²⁵ The
414 observation that PPQ-resistant parasites have a single *Pfmdr1* copy is consistent with this
415 scenario, as maintenance of a single *Pfmdr1* copy (or reversion to a single copy) might avoid
416 importing excessive amounts of PPQ into the digestive vacuole (Appendix_12).^{26,27}

417

418 We note that the association of PPQ resistance with amplification of the *PfPM2-3* cluster on
419 chromosome 14 is not proof of causality. The structured populations of *P. falciparum* parasites
420 in Cambodia²⁸ might confound the robustness of the association and additional loci might also
421 contribute to PPQ resistance. The present findings should be complemented with laboratory
422 investigations of the cellular consequences of this amplification on the parasite response to
423 PPQ and on parasite fitness and transmissibility. Nonetheless, the current data are timely in
424 providing a molecular tool that predicts the appearance of PPQ resistance in endemic settings.

425

426 PPQ is a well-tolerated partner drug currently used in combination with ART derivatives or the
427 ozonide compound arterolane (OZ277).²⁹ The mechanism of PPQ resistance in the specific
428 context of Cambodia, where ART resistance is nearly fixed and drug pressure is high, may not
429 extrapolate to areas where ART resistance has not yet been documented. Nevertheless, we
430 propose to extend the assessment of *PfPM2* gene copy number to areas where PPQ is being
431 used in ACTs at a very large scale, and to combine this assay with K13 sequencing to localize
432 areas of parasite resistance to both components. In Cambodia, where the rapid failure of first-
433 line ACTs is jeopardizing elimination efforts and accelerating the emergence and spread of
434 resistance, the opposing susceptibility between mefloquine and PPQ could be used to
435 implement new strategies based on ACT drug rotation, sequential administration, or triple
436 combinations including both ACT partner drugs. Although challenging to implement, these
437 alternative strategies will help to ensure a long-term efficacy of antimalarials to reach the
438 elimination goal.

439

440 **Contributors**

441 BW, VD, LSR, FA, JCB, DAF, OMP and DM contributed to study design. BS, JB, LM, CB and FA
442 performed analysis of whole-genome sequencing data. NKh, SC, RE, CK, MK, KL and EL
443 performed qPCR and RT-qPCR and LSR performed antibody analysis. NKI, SKe, AD, VD and BW
444 performed in-vitro and ex-vivo drug assays. RL, RH, SKi, and PR gathered clinical data. GN
445 conducted statistical analysis. VD, BW, FA, and DM analysed data. OMP, FA, LSR, DAF and DM
446 wrote the first manuscript. All co-authors have read and approved the final manuscript.

447

448 **Conflict of interest statement**

449 The authors have declared that no conflicting interests exist. PR is a staff member of the World
450 Health Organization. This author alone is responsible for the views expressed in this publication
451 and they do not necessarily represent the decisions, policy or views of the World Health
452 Organization.

453

454 **Acknowledgments**

455 We thank all patients enrolled in the therapeutic efficacy studies, the health centers staff, and
456 the members of the National Center for Parasitology, Entomology and Malaria Control in
457 Cambodia for their support. We are grateful to Dr. Daniel E. Goldberg (Washington University
458 School of Medicine, St Louis, Missouri, USA) for providing anti-Pfplasmepsin2 antibody. This
459 work was supported by the Institut Pasteur in Cambodia, Institut Pasteur Paris, and the World
460 Health Organization. DAF gratefully acknowledges partial funding for this work from the
461 National Institutes of Health (R01 AI109023 and AI124678). LSR is a recipient of a NIH NRSA
462 fellowship F32 AI120578. OMP acknowledges funding from the French Government's
463 Investissement d'Avenir program, Laboratoire d'Excellence "Integrative Biology of Emerging
464 Infectious Diseases" (grant n°ANR-10-LABX-62-IBEID). VD was supported by a doctoral
465 fellowship from the International Division, Institut Pasteur.

466

467 **Research in context**

468 **Evidence before this study**

469 We searched for studies on piperaquine (PPQ) resistance on PubMed using the term
470 "resistance" in combination with "falciparum" and "piperaquine" on May 19, 2016, without any
471 date or language restrictions, and identified 74 publications. This included clinical trials
472 conducted in 11 countries evaluating the efficacy of dihydroartemisinin (DHA)-PPQ for the
473 treatment of uncomplicated *Plasmodium falciparum* malaria (26 reports) or asymptomatic
474 infections (1 report) as well as for intermittent preventive treatment of pregnant women (3
475 reports) or infants (5 reports). In all studies, cure rates were above 90%, except studies
476 conducted in Cambodia after the year 2010, for which cure rates ranging from 85% to 40%
477 were observed. Overall, 26 publications reported susceptibility of parasites collected in 15
478 countries, studied using in-vitro or ex-vivo assays. Virtually all isolates tested by standard dose-
479 response susceptibility assays (using isotopic-, Sybr Green- or HRP2-based parasite
480 quantification) were PPQ-susceptible (<100 nM), except those collected in Cambodia after 2010
481 and samples collected in China before 1998 (when piperaquine monotherapy was intensively

482 used). Piperaquine resistance currently appears confined to Cambodia. It constitutes a major
483 concern, as alternative therapeutic options are scarce and the reduced cure rates translate into
484 prolonged parasite carriage and increased transmission potential of resistant parasites. To map
485 the geographic extension of PPQ resistance and deploy containment measures in order to
486 prevent its further spread, rapid detection tests are needed but are currently lacking. Potential
487 molecular signatures associated with PPQ resistance were investigated in 11 studies. The only
488 consistently observed finding was an increased proportion of single copy *Pfmdr-1* parasites in
489 piperaquine-resistant areas. This is not an informative marker for PPQ resistance, as wild-type,
490 susceptible parasites can also have a single copy *Pfmdr-1 locus*.

491 **Added value of this study**

492 We identified amplification of the *Pfplasmepsin-2-3 (PfPM2-3)* gene cluster encoding proteases
493 involved in hemoglobin degradation as the most significant molecular signature associated with
494 in-vitro resistance to PPQ assessed using the Piperaquine Survival Assays. Using a large
495 longitudinal collection of samples collected during clinical efficacy studies of
496 dihydroartemisinin–PPQ conducted across Cambodia since 2009, we examined 725 *P.*
497 *falciparum* isolates and found that an increased *PfPM2* gene copy number was strongly
498 associated with dihydroartemisinin–PPQ treatment failures. Patients harboring multicopy
499 *PfPM2* parasites had a 20-fold higher risk of recrudescence during the 42-day post-treatment
500 follow up (94% sensitivity and 77% specificity). Our retrospective analysis of samples collected
501 in Cambodia during the last decade before and after introduction of DHA–PPQ as first line
502 treatment showed that the proportion of multicopy *PfPM2* parasites correlated with the
503 increase of DHA-PPQ treatment failure rates, from 2009 to 2015 in western Cambodia and
504 during 2014–2015 in eastern Cambodia. In areas of artemisinin resistance, the clinical efficacy of
505 DHA-PPQ at day 42 fell under 90% when the local proportion of multicopy *PfPM2* parasites rose
506 above 22%.

507 **Implications of all the available evidence**

508 DHA-PPQ failure rates have increased in western Cambodia since 2010 and in eastern
509 Cambodia since 2014. They are caused by parasites that are resistant to both artemisinin and

510 PPQ. Combined analysis of K13 polymorphisms and *PfPM2* copy number represents the first
511 informative molecular signature for DHA-PPQ failures. These molecular markers can now be
512 used to track emergence and dissemination of resistance to artemisinin and PPQ in field
513 populations, especially in areas where PPQ is being or will be recommended in combination
514 with artemisinin derivatives as first-line treatment or in preventive treatment for infants or
515 pregnant women, as currently developed in African settings.

516

517

518 **Figure legends**

519 **Figure 1.** Flow chart of patients enrolled in clinical studies conducted in 2009-2015 in 12
520 provinces across Cambodia to evaluate the efficacy of 3-day DHA-PPQ regimen, and isolates
521 used to detect molecular signatures associated with in-vitro PSA resistance and DHA-PPQ
522 clinical failure.

523

524 **Figure 2.** Manhattan plot showing the significance of copy number variations between whole-
525 genome exome sequences of 23 PPQ-resistant and 8-sensitive culture-adapted lines collected in
526 western Cambodia in 2012 and phenotyped using in-vitro PSA. Each dot represents a gene in
527 the set of 31 culture-adapted parasites, according to chromosome. The x-axis represents
528 genomic location, and the y-axis represents the \log_{10} transformed Wilcoxon test's p-values.
529 After Benjamini-Hochberg correction, only 2 genes, PF3D7_1408000 (*plasmeprin2*) and
530 PF3D7_1408100 (*plasmeprin3*) achieved genome-wide significance ($p=0.03795$).

531

532 **Figure 3.** Ex-vivo PSA survival rates and single ($n=67$) and multicopy *PfPM2* ($n=67$) as estimated
533 by qPCR in isolates collected before DHA-PPQ treatment stratified by K13 genotype. Patients
534 were enrolled in clinical studies conducted in 2014-2015 in Mondulkiri, Rattanakiri, Siem Reap
535 and Stung Treng provinces, stratified by K13 genotypes. K13 polymorphisms were detected in
536 65/69 PPQ-resistant (64 C580Y, 1 Y493H) and 17/65 PPQ-susceptible isolates (15 C580Y, 1
537 C469F and 1 A626E). Three parasite lines with discordant data were observed: two resistant

538 lines with non-amplified *PfPM2* and *PfPM3* loci (6246 and 6395) and one sensitive line with 2
539 *PfPM2* copies (6369) (see Table 2).

540
541 **Figure 4.** Cumulative proportion of non-recrudescent patients treated with a 3-day course of
542 DHA-PPQ according to (A) *PfPM2* gene copy number (Logrank test: overall p<0.0001; p<0.0001
543 for single copy vs. 2 copies; p<0.0001 for single copy vs. ≥3 copies; p=0.017 for 2 copies vs. ≥3
544 copies) and (B) *PfPM2* gene copy number and K13 genotype (Logrank test: overall p<0.0001;
545 p<0.0001 for *K13* wild-type/*PfPM2* single copy vs. *K13* wild-type/*PfPM2* multi-copy; p=0.002 for
546 *K13* wild-type/*PfPM2* single copy vs. *K13* mutant/*PfPM2* single copy; p<0.0001 for *K13* wild-
547 type/*PfPM2* single copy vs. *K13* mutant/*PfPM2* multi-copy; p=0.001 (HR=6.9 [95%CI: 0.5-96.6]),
548 for *K13* wild-type/*PfPM2* multicopy vs. *K13* mutant/*PfPM2* single copy; p=0.07 (HR=2.6 [95%CI:
549 1.3-5.5]), for *K13* wild-type/*PfPM2* multicopy vs. *K13* mutant/*PfPM2* multi-copy; p<0.0001
550 (HR=17.5 [95%CI: 12.2-25.2]), for *K13* mutant/*PfPM2* single copy vs. *K13* mutant/*PfPM2* multi-
551 copy) detected in isolates collected at the time of enrollment, prior to treatment.

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1 **Table 1.** Proportion of PCR-corrected *P. falciparum* recrudescences observed at day 42 in 2009-
 2 2015 in 12 provinces across Cambodia in patients treated with a 3-day course of DHA-PPQ
 3 regimen. Site location and years of collection are provided for isolates with in-vitro and ex-vivo
 4 PSA profiles (the map of the location of the study sites is presented Appendix_1).

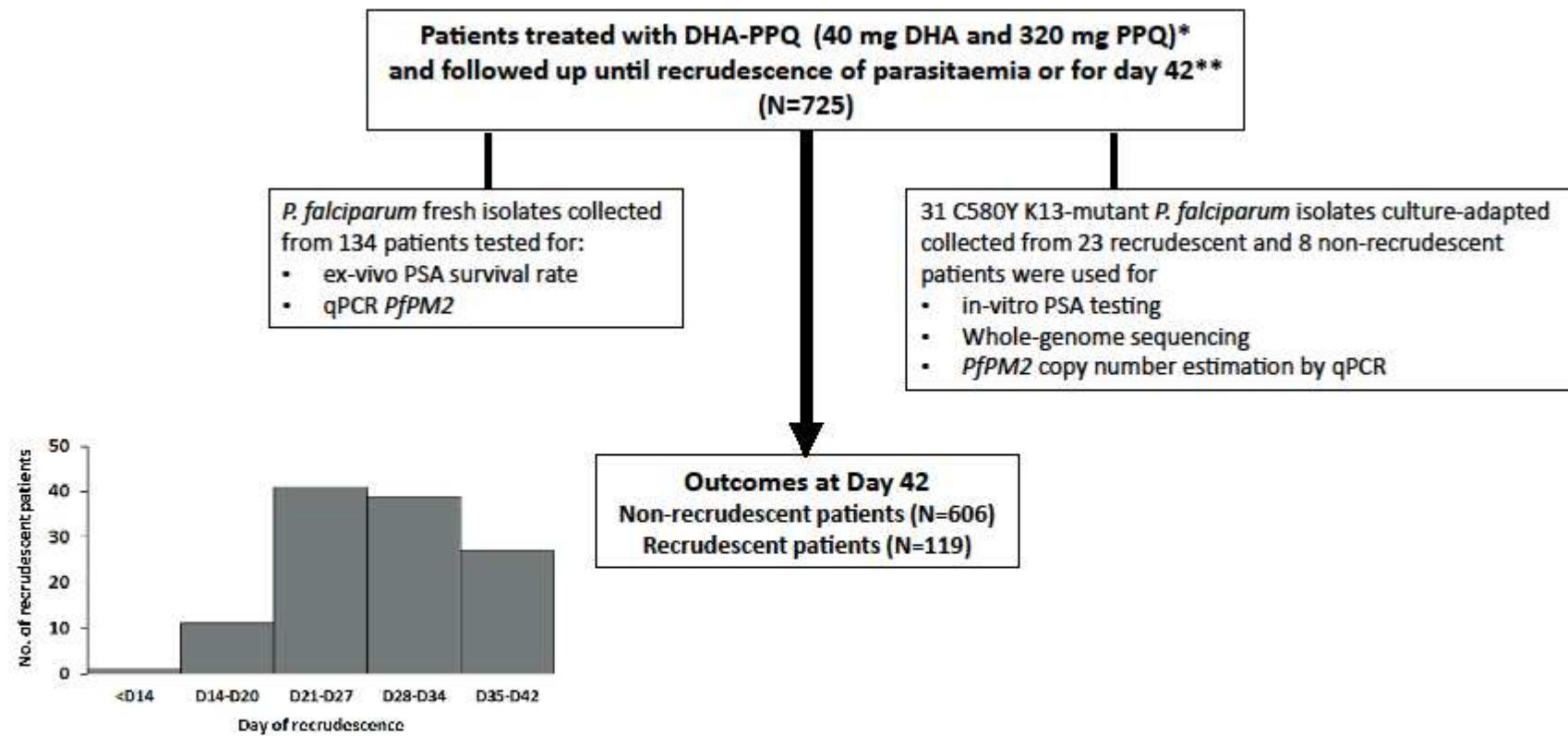
Year	Site (province)	No. patients treated and followed up	No.of patients classified as recrudescent (PCR-corrected) (%)	No. isolates with in-vitro PSA survival data	No. isolates with ex-vivo PSA survival data
2009	Pailin	32	3 (9.4)	0	0
	Preah Vihear	30	0 (0)	0	0
2010	Pailin	21	4 (19.0)	0	0
	Pursat	32	3 (9.4)	0	0
2011	Rattanakiri	30	0 (0)	0	0
	Kratié	51	2 (3.9)	0	0
	Preah Vihear	34	2 (5.9)	0	0
2012	Pursat	41	7 (17.1)	0	0
	Battambang	39	12 (30.8)	19	0
	Kampong Speu	22	0 (0)	4	0
	Kampong Thom	38	0 (0)	2	0
2013	Pursat	23	2 (8.7)	6	0
	Kampot	17	1 (5.9)	0	0
	Kratié	22	0 (0)	0	0
	Preah Vihear	16	1 (6.3)	0	0
2014	Rattanakiri	31	1 (3.2)	0	0
	Mondulkiri	39	4 (10.3)	0	0
	Siemreap	40	25 (62.5)	0	0
	Stungtreng	33	11 (33.3)	0	0
2015	Rattanakiri	34	5 (14.7)	0	34
	Mondulkiri	16	4 (25.0)	0	16
	Rattanakiri	54	16 (29.6)	0	54
	Siemreap	17	10 (58.8)	0	17
	Stungtreng	13	6 (46.1)	0	13
	Total	725	119 (16.4)	31	134

1 **Table 2.** Details of the 31 K13-C580Y mutant (and the 3D7 reference line), PPQ-resistant and -
 2 sensitive culture-adapted parasites analyzes by whole-genome sequencing. The last column
 3 lists the DNA expansion types observed in the region of chromosome 14 encoding the
 4 plasmepsin 1-4 hemoglobinas.

Parasite lines ID	Year	Site location	in-vitro PSA survival rate (%)	in-vitro susceptibility to PPQ*	DNA expansion type**
3D7	-	-	0.1	Sensitive	No amplification
6273	2012	Kampong Speu	0.2	Sensitive	No amplification
6337	2012	Kampong Speu	0.4	Sensitive	No amplification
6403	2012	Pursat	0.5	Sensitive	No amplification
6267	2012	Kampong Speu	0.5	Sensitive	No amplification
6349	2012	Kampong Thom	0.6	Sensitive	No amplification
6237	2012	Kampong Thom	0.8	Sensitive	No amplification
6410	2012	Battambang	6	Sensitive	No amplification
6369	2012	Pursat	6.4	Sensitive	Type 2
6395	2012	Battambang	19.2	Resistant	No amplification
6341	2012	Pursat	25.8	Resistant	Type 2
6280	2012	Battambang	28.9	Resistant	Type 2
6246	2012	Kampong Speu	36.9	Resistant	No amplification
6293	2012	Battambang	39.3	Resistant	Type 2
6391	2012	Battambang	39.4	Resistant	Type 1
6272	2012	Battambang	40	Resistant	Type 2
6218	2012	Battambang	40.8	Resistant	Type 1
6302	2012	Battambang	42.5	Resistant	Type 1
6229	2012	Battambang	46.6	Resistant	Type 1
6443	2012	Battambang	49.6	Resistant	Type 1
6430	2012	Battambang	51.3	Resistant	Type 1
6429	2012	Pursat	51.8	Resistant	Type 1
6365	2012	Battambang	51.8	Resistant	Type 2
6394	2012	Battambang	56.7	Resistant	Type 1
6219	2012	Battambang	58.6	Resistant	Type 3
6408	2012	Battambang	58.7	Resistant	Type 3
6224	2012	Pursat	61.4	Resistant	Type 1
6431	2012	Battambang	61.5	Resistant	Type 1
6320	2012	Battambang	62.1	Resistant	Type 1
6261	2012	Pursat	70.5	Resistant	Type 1
6411	2012	Battambang	71.6	Resistant	Type 1
6427	2012	Battambang	77.4	Resistant	Type 3

5 * Threshold used to define in-vitro susceptibility to PPQ: sensitive if survival rates were <10%
 6 and resistant if survival rates were ≥10%.

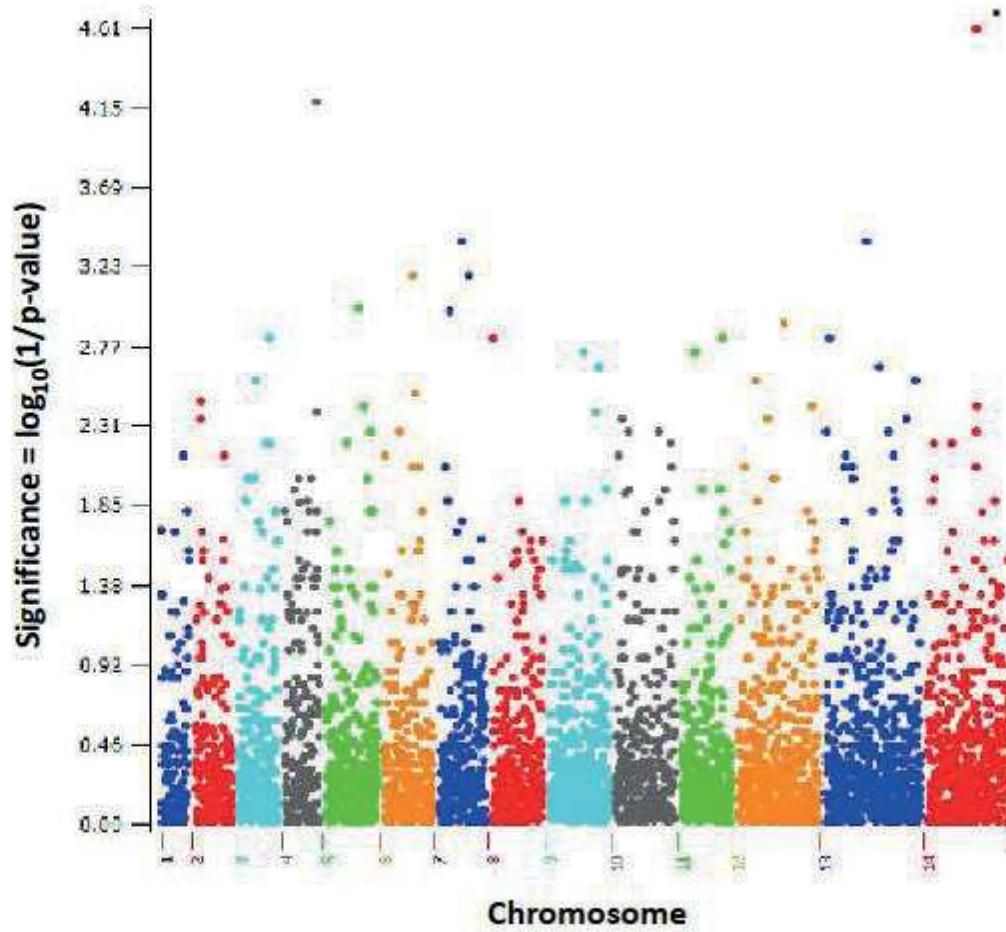
7 ** See Appendix_6 for details

Figure 1

* Supervised DHA-PP was administered once daily for 3 days (day 0, 24h, 48 h). Dosing was based on body weight, as follows: (i) <19 kg= 40mg DHA–320mg PPQ/day; (ii) 19-29kg= 60mg DHA–480mg PPQ/day; (iii) 30-39 kg= 80mg DHA–640mg PPQ/day; (iv) >40 kg= 120mg DHA–960mg PPQ/day. For children unable to swallow tablets, DHA-PP was dissolved in 5 ml of water. Patients were observed for 1 h post-dosing and were re-dosed with a full or half dose if vomiting occurred within 30 min or between 31 and 60 min, respectively. Those who vomited after the second dose were withdrawn from the study and were given parenteral rescue treatment (intramuscular artemether). Patients with axillary temperatures of 37.5°C were treated with paracetamol. Patients were seen daily to day 3 and then weekly for 6 weeks (day 42) for clinical examinations (axillary temperature, symptom check) and malaria blood films. Home visits were conducted if patients failed to come back for their follow-up appointments.

** Were excluded from the analysis, withdrawn patients, patients lost to follow-up, patients classified as reinfected (based on *msp1*, *msp2* and *glurp* genotypes)

Figure 2



* Wilcoxon test $p=0.139$ (Bonferroni correction) and $p=0.03795$ (Benjamini-Hochberg correction)

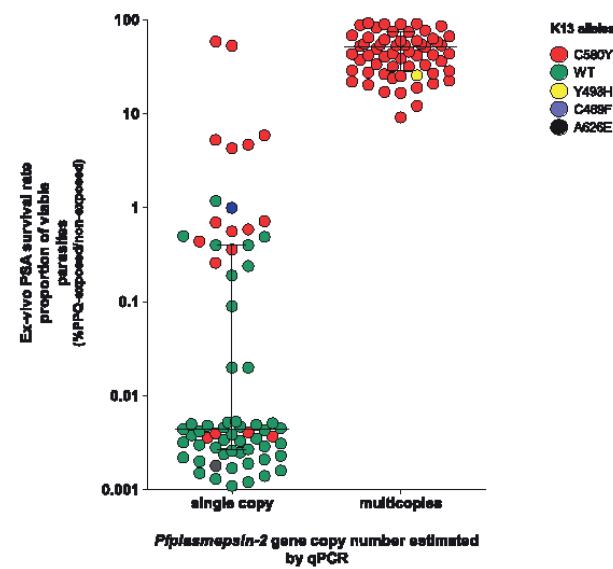
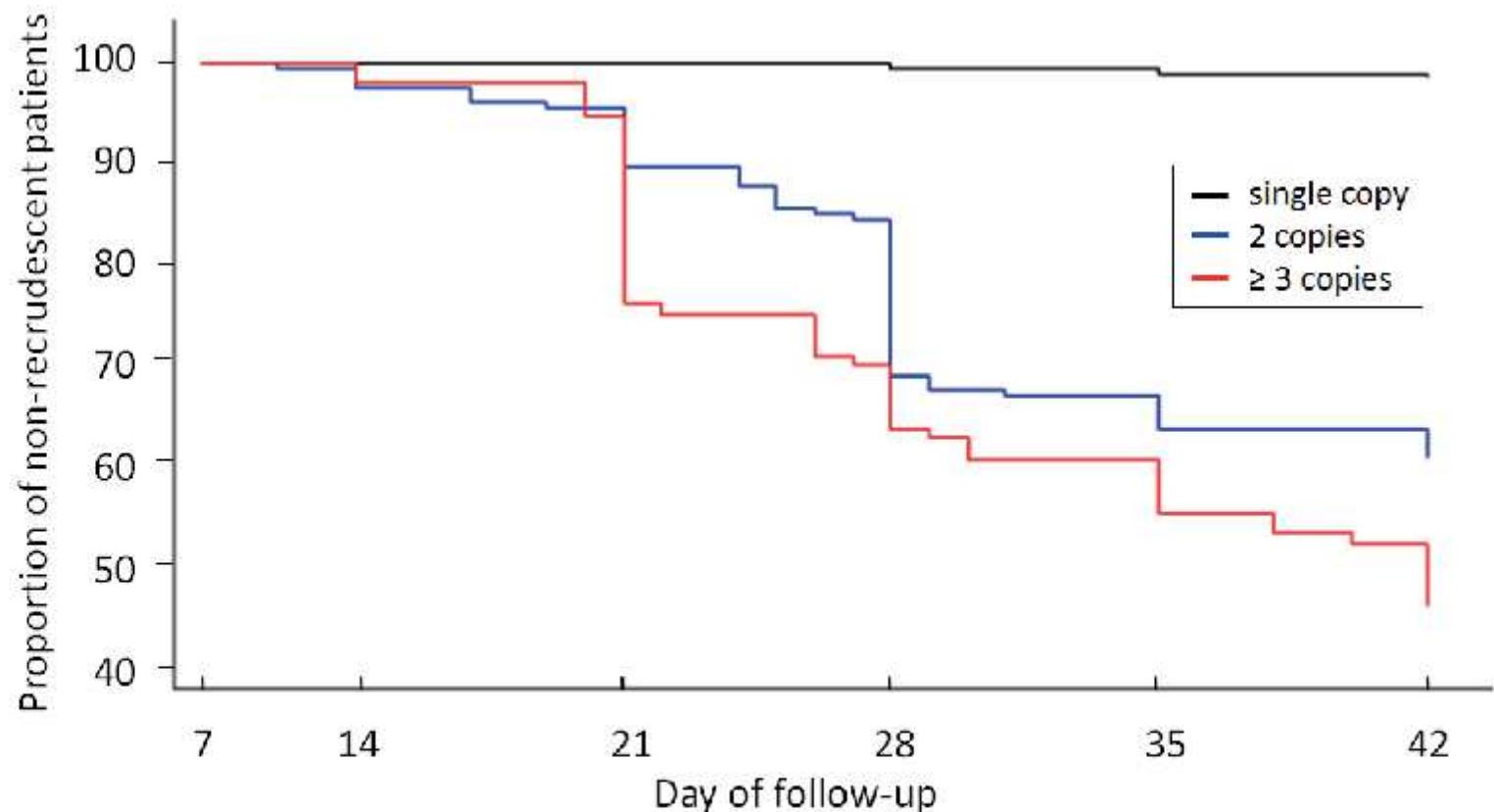
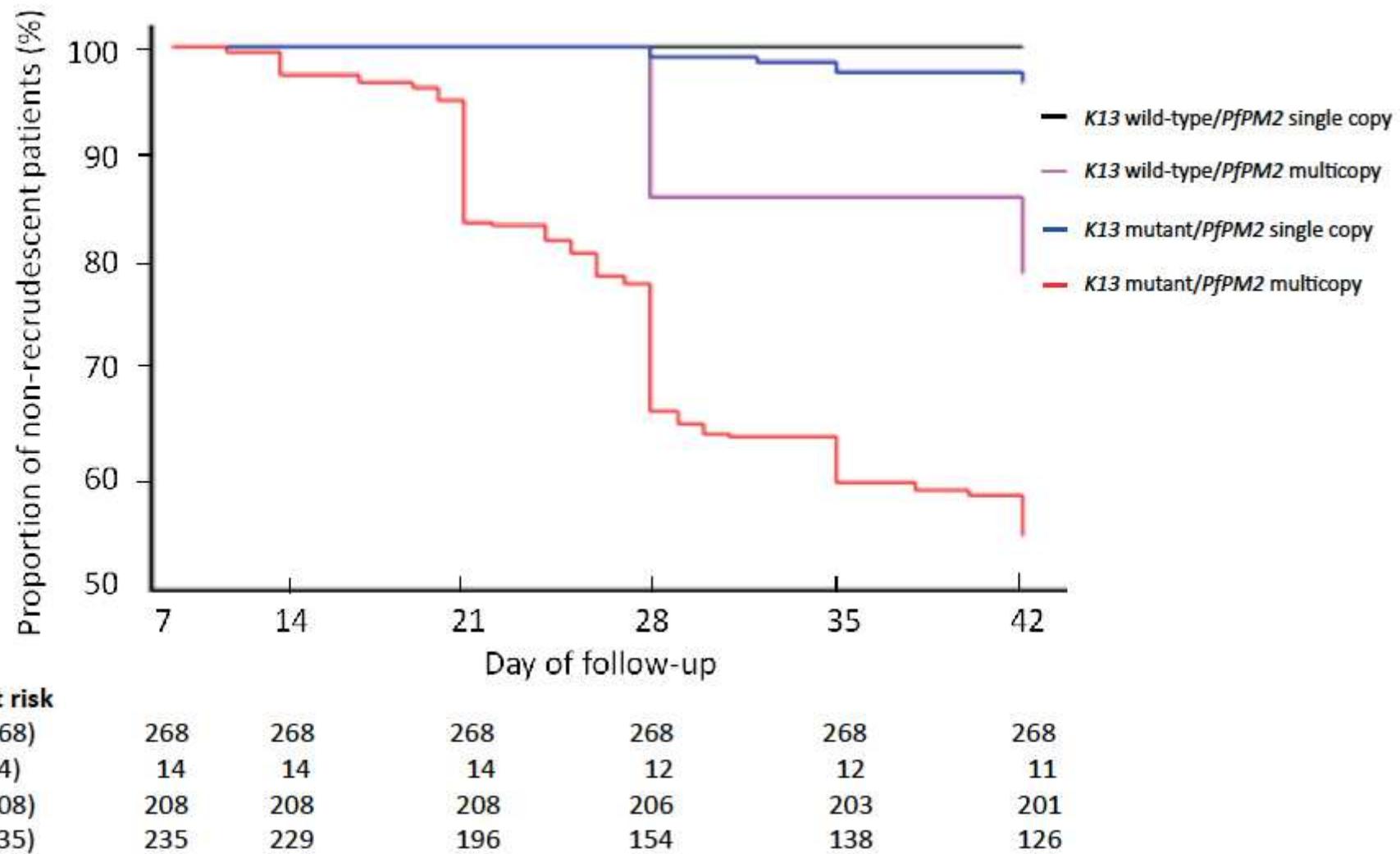
Figure 3

Figure 4A



Number at risk

	7	14	21	28	35	42
single copy (D0=476)	476	476	476	474	471	469
2 copies (D0=153)	153	149	137	105	97	93
≥ 3 copies (D0=96)	96	94	73	61	53	44

Figure 4B

Articles supplémentaires

J'ai également participé de manière plus anecdotique à deux études ayant fait l'objet de publications présentées en annexe de cette thèse.

La première publication (Article annexe a) présente la validation d'un nouvel allèle du gène *K13* comme marqueur moléculaire de la résistance à l'ART en Asie du Sud-Est, l'allèle N458Y. L'OMS demande en effet que chaque nouvelle mutation soit validée comme marqueur moléculaire de la résistance à l'ART selon l'une des méthodes suivantes :

- corrélation avec une clairance parasitaire retardée dans les études cliniques ;
- sensibilité réduite en test phénotypique *ex vivo* ou *in vitro* (tel que le RSA) ;
- sensibilité *in vitro* réduite dans des études de transfection impliquant l'insertion de l'allèle K13 à valider.

L'allèle N458Y a été validé à l'aide des deux premières méthodes (clairance parasitaire ≥ 5 heures et taux de survie en RSA $\geq 1\%$). À l'inverse, la mutation A578S évoquée dans l'article 3 a pu être invalidée grâce à la troisième méthode.

La seconde publication (Article annexe b) décrit le développement d'un algorithme dédié à la détection de variations du nombre de copies dans l'exome entier de *Plasmodium falciparum*. Cet algorithme est basé sur une stratégie adaptée de lecture en profondeur de données issues de Next Generation Sequencing (NGS) et s'intitule PlascoCNVScan. C'est cet algorithme qui nous a permis d'identifier les variations du nombre de copies des gènes *PfPM2* et *PfPM3* présentées dans l'article 6 comme marqueurs moléculaires candidats de la résistance à la PPQ.

Revue bibliographique

Les résultats présentés dans les articles 1 à 5 ont fait l'objet d'une revue bibliographique dans laquelle nous détaillons les avancées récentes dans notre compréhension des résistances à l'ART et à la PPQ et discutons des défis auxquels la communauté scientifique devra faire face dans les années à venir.

Revue bibliographique 7

Plasmodium falciparum resistance to artemisinin derivatives and piperaquine: a major challenge for malaria elimination in Cambodia

Valentine Duru, Benoit Witkowski, Didier Ménard

The American Journal of Tropical Medicine and Hygiene, accepté

1 DURU AND OTHERS

2 RESISTANCE TO ART AND PPQ

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19 ***Plasmodium falciparum* resistance to artemisinin derivatives and**
20 **piperaquine: a major challenge for malaria elimination in Cambodia**

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28 Word count: Total (5130), Abstract (197 words), text (4933 words)

29 No conflict of interest

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44 **Keywords:** Malaria, Drug resistance, Artemisinin derivatives, Piperaquine, Elimination

45 **Abstract**

46 Artemisinin-based combination therapies (ACTs) are the cornerstone of current strategies for
47 fighting malaria. Over the last decade, ACTs have played a major role in decreasing malaria
48 burden. However, this progress is being jeopardized by the emergence of artemisinin-resistant
49 *Plasmodium falciparum* parasites. Artemisinin resistance was first detected in western
50 Cambodia in 2008 and has since been observed in neighboring countries in Southeast Asia.
51 The problem of antimalarial drug resistance has recently worsened in Cambodia, with reports
52 of parasites resistant to piperaquine, the latest generation of partner drug used in combination
53 with dihydroartemisinin, leading to worrying rates of clinical treatment failure. The monitoring
54 and the comprehension of both types of resistance are crucial to prevent the spread of multi-
55 drug resistant parasites outside Southeast Asia, and particularly to Africa, where the public
56 health consequences would be catastrophic. To this end, new tools are required for studies of
57 the biological and molecular mechanisms underlying resistance to antimalarial drugs and for
58 monitoring the geographic distribution of the resistant parasites.

59 In this review, we detail the major advances in our understanding of resistance to artemisinin
60 and piperaquine and define the challenges that the malaria community will have to face in the
61 coming years.

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72 **Background**

73 In 2016, malaria remains a major human parasitic disease worldwide, affecting almost 3.2
74 billion people in 108 countries¹. *Plasmodium falciparum*, the most lethal human malarial
75 pathogen, is responsible annually for 214 million cases and 438,000 deaths¹. Over the last
76 decade, the malaria mortality rate has decreased considerably (by 48% worldwide), mostly due
77 to the reinforcement of malaria control efforts and increases in the financial resources
78 provided by both public and private international organizations. The prevention of malaria
79 transmission, and the early detection and prompt treatment of malaria cases are currently the
80 cornerstones of malaria control and elimination strategies. Malaria prevention is based on
81 vector control measures (e.g. use of nets impregnated with long-lasting insecticide and the
82 spraying of insecticides indoors) to limit contact between humans and vectors, thereby
83 decreasing malaria transmission and morbidity. The management of suspected cases of
84 malaria seen at health facilities or in the community is based on the early biological detection
85 of malaria parasites by microscopy or rapid diagnostic tests, followed by effective and prompt
86 treatment of the confirmed cases. These approaches limit the duration of the disease and
87 prevent severe complications and death. Despite intensive efforts to develop effective vaccines
88 and the promising results reported by vaccine trials (e.g. RTS,S/AS01 vaccine)², it will be several
89 years before these new preventive weapons can be used in clinical practice.

90 Over the last century, all the antimalarial drugs deployed worldwide have inevitably led to the
91 selection and spread of drug-resistant *P. falciparum* parasites, particularly in Southeast Asia
92 (Figure 1). In the 1950 and 1960s, the WHO Global Malaria Eradication Program was based on
93 the massive deployment of chloroquine, along with vector control measures (DDT). This program
94 was initially highly successful, but the progress made was rapidly compromised by the
95 emergence and spread of chloroquine-resistant parasites and DDT-resistant vectors. Later, in the
96 1970s and 1980s, a similar pattern was observed for alternative antimalarial drugs, such as
97 sulfadoxine-pyrimethamine, which was used in areas in which chloroquine resistance had

98 developed, leading to the selection of parasites resistant to anti-folate agents. At the end of the
99 1990s^{3, 4}, the global health situation, particularly in Sub-Saharan Africa, was critical, with more
100 than three million deaths annually due to malaria^{5, 6}. In their attempts to control this alarming
101 situation, stakeholders began to take an interest in derivatives of artemisinin, a sesquiterpene
102 lactone discovered in 1972 by Chinese scientists during a program of research involving the
103 screening of compounds from plants (Military code name: Project 523). Artemisinin derivatives,
104 which are extracted from *Artemisia annua*, have been used to treat acute malaria in traditional
105 Chinese medicine for more than two thousand years. Novartis developed and patented the first
106 artemisinin-based combination therapy (ACT), consisting of a combination of artemether and
107 lumefantrine. The massive deployment of this new treatment in South Africa in 2000-2001 along
108 with vector control measures was highly successful, decreasing the number of deaths from
109 malaria by 87.5% in less than one year⁷. In 2001, the WHO recommended the use of ACT as a
110 first-line treatment for uncomplicated malaria in areas in which the parasites were resistant to
111 monotherapy with other drugs, such as mefloquine⁸. This recommendation was extended to the
112 entire world in 2006, and the WHO banned the use of artemisinin in monotherapy to prevent the
113 selection of artemisinin-resistant parasites. In 2012, five different ACTs, including
114 dihydroartemisinin-piperaquine, artesunate-mefloquine, artemether-lumefantrine, artesunate-
115 sulfadoxine-pyrimethamine and artesunate-amodiaquine were deployed in 79 countries with
116 endemic malaria. At present, successful malaria control and, to a greater extent, malaria
117 elimination, rely strongly on effective anti-malarial treatments. As no alternative to artemisinin
118 derivatives are ready for deployment before 2020, emergence of resistance to artemisinin
119 derivatives and partner drugs represents a major threat to malaria control and elimination efforts
120 worldwide.

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124 **Resistance to artemisinin derivatives: past and present research challenges**

125 **Clinical phenotype.** Partial resistance to artemisinin in *P. falciparum* was first reported in
126 2008, in Battambang province in western Cambodia⁹. It was subsequently confirmed in 2009,
127 in Pailin province, the well-known epicenter of antimalarial multidrug resistance¹⁰. Artemisinin
128 resistance has since been reported elsewhere in western Cambodia, western Thailand,
129 southern Myanmar (Burma) and southern Vietnam^{11, 12, 13, 14}, and in China¹⁵.

130 Artemisinin resistance is defined clinically as delayed parasite clearance^{16, 17, 18} and a half-life
131 for parasite clearance ≥ 5 hours has been associated with artemisinin resistance in Southeast
132 Asia¹⁸. In high-transmission settings, such as Sub-Saharan Africa, where the partial immunity
133 of adults plays a significant role in parasite clearance, the threshold need to be defined^{19, 20}.
134 Currently, this approach is considered to be the gold standard, but suffers from several
135 drawbacks: firstly, parasite clearance studies are both logistically and financially demanding,
136 requiring the screening of thousands of febrile individuals to identify the small number of
137 patients who will be hospitalized for several days; secondly, parasite clearance rates depend
138 on many factors other than the intrinsic drug resistance of the parasites, relating to drug
139 administration, immunity, and pharmacodynamics. Alternative *in vivo* approaches has been
140 proposed. For instance, Beshir *et al*²¹ assessed the association between parasite clearance
141 following ACT and transmissibility in mosquitoes by membrane-feeding assays. They found
142 that Kenyan children treated with a standard 3-day ACT course had commonly a persistent
143 residual parasitemia (assessed by qPCR), associated with longer duration of gametocyte
144 carriage and a higher likelihood of infecting mosquitoes. However, even being innovative and
145 elegant, this approach is logistically demanding and not adapted for high scale application.

146 Before 2013, the principal alternative approach for studies of artemisinin resistance in *P.*
147 *falciparum* was the determination, in classical assays, of IC₅₀ values (the concentration
148 inhibiting parasite growth by 50%) for dihydroartemisinin. However, all *in vitro/vivo* studies,
149 except one⁹ have reported no significant correlation¹¹ between clinical outcome (half-life for

150 parasite clearance) and dihydroartemisinin IC_{50s}. One potential reason for this observation was
151 later put forward²²: the parasites in these assays were exposed to very low concentrations of
152 dihydroartemisinin (≤ 64 nM) for 48–72 h, whereas parasites *in vivo* are exposed to higher
153 concentrations of drugs for only 1–2 h.

154 **Rethinking *in vitro* phenotype.** A novel assay to overcome this problem was developed in
155 2012–2013. This assay was based on the exposure of culture-adapted or fresh isolates of *P.*
156 *falciparum* parasites to the physiological conditions encountered in humans: *P. falciparum*
157 parasites were exposed to 700 nM dihydroartemisinin (the active metabolite of all
158 artemisinins) for 6 hours, reflecting typical doses and exposure times for ACTs. This assay
159 (named 'Ring-stage Survival Assay', RSA) successfully detected significant differences in
160 susceptibility *in vitro* between parasites originating from areas in which clinical resistance to
161 artemisinin had and had not been observed²². *In vitro* artemisinin susceptibility was found to
162 be stage-dependent and strongly linked to the decrease in susceptibility of early ring-stages.
163 This further explains the lack of correlation between clinical outcome and dihydroartemisinin
164 IC_{50s}, during which the parasites are exposed to the drug throughout their cycle. These parasite
165 stages were capable of surviving to survive short periods of drug exposure by entering a state
166 of developmental arrest (dormancy)^{23, 24}. This observation was consistent with the findings of
167 previous transcriptome studies showing that artemisinin-resistant parasites had a particularly
168 long ring-stage and lower metabolic activities¹⁸ and were also consistent with those of
169 modeling studies suggesting a significant decrease in the efficacy of artemisinin derivatives
170 against ring-stage parasites in western Cambodia as early as 2007–2008²⁵. Survival rates in the
171 RSA were also strongly correlated with clinical phenotype (half-life for parasite clearance)^{22, 26}.
172 **A molecular marker.** The molecular signatures associated with resistance to artemisinins
173 were then investigated by sequencing the exome of an African strain (F32-Tanzania) that
174 became resistant to artemisinin after repeated exposure to increasing concentrations of the
175 drug over a period of five years (F32-ART)²⁷. The exome of F32-ART was compared with that
176 of its sibling clone (F32-TEM) cultured under the same conditions but without the drug. A

177 detailed analysis of the differences between the two exomes identified eight non-synonymous
178 SNPs in seven genes. Analysis of the genomes of three intermediate strains (after 22, 40 or 56
179 cycles of exposure) and of 49 strains isolated in Cambodia made it possible to determine the
180 chronological order in which these mutations appeared. This analysis revealed a strong
181 association between mutations in the propeller domain of the *Kelch* gene located on
182 chromosome 13 (K13, PF3D7_1343700) and *in vitro* susceptibility to artemisinin in the RSA.
183 Previous GWAS studies performed by 2 independent groups identified this region as
184 associated with delayed parasite clearance half lives^{28, 29}. A study of the temporal (2001-2012)
185 and spatial distribution of K13 mutant parasites showed that they had progressively spread in
186 zones classified as 'resistant' (western Cambodia) but not in areas in which artemisinin
187 resistance had never been observed (eastern Cambodia). The detection of parasites with K13
188 mutations in patients with slow rates of parasite clearance confirmed that this molecular
189 marker was a major determinant of resistance to artemisinin derivatives³⁰. Definitive evidence
190 was later obtained in site-specific genome editing experiments using zinc-finger nucleases³¹
191 or the CRISPR-Cas9 system³². This series of experiments showed that the replacement of a
192 wild-type K13 allele in parasites susceptible to artemisinin by different K13 mutant alleles
193 (Y493H, C580Y, M476I, R539T, I543T) conferred artemisinin resistance (survival rates assessed
194 using the RSA increased from ≤1% to 2% to 29% after the insertion of K13 mutations^{32, 33}),
195 while the removal of K13 mutations in isolates from Cambodia had the opposite effect (survival
196 rates decreased from 13 to 49% to 0.3 to 2.4% after the removal of K13 mutations³³).
197 This discovery provided unprecedented opportunities for improving strategies for the
198 monitoring of artemisinin resistance. Several molecular epidemiology studies, mostly in
199 Southeast Asia^{30, 34, 35, 36, 37}, China^{38, 39, 40}, Indian subcontinent^{41, 42} and Africa^{4, 18, 43, 44, 45, 46, 47, 48, 49,200 50}, investigated the frequency of K13 mutant alleles, their spatial distribution and, in some
201 cases, their association with clinical artemisinin resistance (day 3 positivity rate or parasite
202 clearance half-life). Only 13 independent K13 mutations of about 100 tested were found to be
203 associated with clinical resistance, with evidence of the independent emergence of the same

mutation in different areas. Only four of these mutations, all from Asia (C580Y, R539T, I543T and Y493H), have been validated *in vitro*. A worldwide map of *Plasmodium falciparum* K13-propeller polymorphism was recently published from a study involving 41 partner institutions and 14,037 samples collected from 163 sites (in 59 countries in which malaria is endemic), providing a global overview of the distribution of artemisinin resistance and fundamental data for drug policy-makers and for future surveillance activities⁵¹. The main findings of the KARMA study (K13 Artemisinin Resistance Multicenter Assessment) suggest that artemisinin resistance remains confined to Southeast Asia (including Cambodia, PDR of Laos, Thailand, Vietnam and Myanmar) and China. In these two regions, the proportions of parasites with K13 mutations were estimated at 51.8% (95% CI: 48.5-55.3%) and 25.6% (95% CI: 19.3-33.3%), respectively. Two independent foci of emergence were identified — one in Cambodia-Vietnam-PDR of Laos and the other in eastern Thailand-Myanmar-China — with no evidence of parasite flow between the two foci, consistent with other reports which showed that most K13 mutations emerged independently from the same foci in Southeast Asia, with the exception of mutations C580Y and Y493H, which probably emerged in Cambodia and subsequently spread to Lao PDR and Vietnam³⁵. Conversely, no K13 mutant alleles were found in South America, Oceania, the Philippines and some of the countries in Central Asia. In Africa, the frequency of K13 mutant alleles (<1.5%) was low. None African K13 mutant alleles were associated with artemisinin resistance and none of the Asian mutants known to be associated with resistance to artemisinin (C580Y, R539T, I543T and Y493H) were found among the 9,542 African samples tested. The most frequent mutation in Africa, the A578S mutation (3.8%), which was previously detected in India, Bangladesh and Thailand, was susceptible in the *in vitro* RSA^{0-3h} and had no effect on positivity rate on day 3. Thus, not all non-synonymous K13 mutants are associated with artemisinin resistance. The effective surveillance of artemisinin resistance must therefore be based on a combination of molecular data (genotyping to detect K13 mutants and haplotyping of the neighboring loci to assess the spread of mutant parasites carrying the same K13 mutation) with clinical and *in vitro* studies (to validate the association of the mutations

231 detected with artemisinin resistance). It is also worth noting that the lack of association
232 between ACT treatment failures and K13 polymorphisms in Africa⁵² could be a sign of K13-
233 independent mechanisms which need to be investigated. There is also a possibility that a
234 particular genetic backbone is mandatory to increase the level of resistance conferred by K13
235 mutations, and this specific genetic background is for now only found in Asian parasite
236 populations⁵³.

237 ***Insight into resistance mechanisms.*** Despite the immense progress of recent years, the
238 mechanisms involved in *P. falciparum* resistance to artemisinin derivatives remain poorly
239 understood. Such an understanding is essential for the definition of new effective strategies
240 to prevent the spread of resistant strains, and for the development of new detection tools and
241 new drugs targeting these mechanisms. The protein encoded by the K13 gene has a BTB/POZ
242 domain (PDB 4YY8) and a C-terminal helical Kelch domain. The mutations associated with
243 artemisinin resistance affect one of the β-sheets of the Kelch domain (Y493H in blade 2, R539T
244 and I543T in blade 3 and C580Y in blade 4). Such domains are frequently involved in protein-
245 protein interactions and the validated mutations may alter the overall structure of the protein,
246 substantially modifying the interactions between K13 and its potential partner proteins.
247 Mbengue *et al.* recently provided new insight into the mechanisms involved in K13-mediated
248 artemisinin resistance⁵⁴. They demonstrated that the presence of the C580Y mutation
249 decreased polyubiquitination of the *P. falciparum* phosphatidylinositol-3-kinase (PfPI3K,
250 which is strongly inhibited by artemisinins) by modifying its binding to K13, thereby limiting
251 PfPI3K proteolysis and increasing production of the phosphatidylinositol-3-phosphate (PI3P).
252 PI3P levels were found to be predictive of artemisinin resistance in both clinical and genetically
253 modified *P. falciparum* strains, with high PI3P levels associated with an increase in artemisinin
254 resistance in the absence of K13 mutation.

255 Moreover, the K13 protein displays similarity to the human KEAP1 protein involved in the
256 oxidative stress response mediated by the transcription factor Nrf2^{55, 56, 57, 58, 59}. Mutations of
257 the KEAP1 gene impairing the binding of the encoded protein to Nrf2 have been found in

258 human cancers resistant to chemotherapy. This resistance is thought to be due to the diffusion
259 of NrF2 in the nucleus and the activation of several genes involved in the antioxidant response
260 (AREs), promoting cell survival⁶⁰. A similar mechanism may account for the resistance of *P.*
261 *falciparum* to artemisinin. It is therefore important to screen for proteins interacting with K13,
262 to identify the cellular pathways involving K13 and to understand how these pathways are
263 modified by K13 mutations.

264 **Perspectives.** Major advances have been made since the detection of the first case of
265 artemisinin resistance in 2008, and the identification of the K13 mutation as a major
266 determinant of artemisinin resistance has made it possible to conduct large-scale molecular
267 monitoring programs with simple, inexpensive techniques (e.g. extraction of DNA from finger-
268 prick blood samples on filter paper and its use for the detection of mutations by PCR and
269 sequencing). Such programs can be used to monitor resistance in real time, making it possible
270 to adapt antimalarial treatments according to the epidemiological situation in a given area.
271 The timely implementation of such initiatives should make it possible to avert public health
272 disasters like the spread of chloroquine-resistant parasites in Africa in the 1980s. Studies of
273 the K13 gene may also shed light on the cellular and molecular mechanisms underlying
274 resistance to artemisinin derivatives, thereby making it possible to limit the spread of this
275 resistance phenotype through the development of appropriate new drugs. However, as
276 artemisinin derivatives are systematically administered with a partner drug, it is important to
277 understand the mechanisms involved in the emergence of parasites resistant to partner drugs,
278 particularly in areas of artemisinin resistance, in which the clearance of parasites from the
279 bloodstream of patients depends primarily on the efficacy of the partner drug.

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281 **Resistance to piperaquine: research challenges**

282 For decades, the Thai-Cambodian border has been the epicenter of multidrug-resistant *P.*
283 *falciparum* malaria emergence. This region saw successively the demise of chloroquine

284 (1960s), sulfadoxine-pyrimethamine (1970s), and mefloquine (1990s) treatments³. Mefloquine,
285 initially introduced in the 1990s, was replaced, in 2000, by artesunate-mefloquine (AS-MQ) in
286 Cambodia⁶¹. The clinical efficacy of this combination decreased rapidly, from 90.1% in 2001 to
287 79.4% in 2004⁶², probably due to the high proportion of mefloquine-resistant parasites (high
288 mefloquine IC₅₀ values and amplified *Pfmdr1* gene copy)^{63, 64, 65}. AS-MQ was replaced by
289 dihydroartemisinin-piperaquine (DHA-PPQ)^{66, 67} in 2008, initially in western Cambodia (Pailin
290 province) and then throughout the entire country in 2010, as the first-line drug of choice for
291 uncomplicated malaria⁶⁸. Compelling data, presented in Table 1 and Figure 2, clearly illustrate
292 that the decrease of the ACTs efficacy is always emerging in western Cambodia before to
293 spread eastwards. It is worth to note that AS-MQ regained efficacy promptly after it was
294 replaced by DHA-PPQ (for example, in Battambang, the reported efficacy of AS-MQ in 2004
295 was only 79.3% but reaches 100% in 2010, one year only after the implementation of DHA-
296 PPQ as a first-line treatment in this area), suggesting that a switch from DHA-PPQ to AS-MQ
297 could be a temporary solution in areas where DHA-PPQ is now failing. Clinical studies
298 assessing artesunate-pyronaridine (ATS-PYRO) efficacy conducted in Cambodia were
299 disappointing: indeed, ATS-PYRO did not meet the World Health Organization efficacy criteria
300 for first-line treatment of *P. falciparum* malaria in western Cambodia (i.e., >90% PCR-adjusted
301 ACPR at day 42). This was largely unexpected, given the high efficacy of this ACT in other Asian
302 countries and in Africa^{69, 70, 71, 72, 73}. The reasons of this disparity remain unclear, but cross-
303 resistance between pyronaridine and other antimalarial drugs like piperaquine should be
304 investigated.

305 **DHA-PPQ is failing in Cambodia.** The most recent clinical studies conducted in Cambodia
306 between 2012 and 2015 have reported an alarming increase in the proportion of late clinical
307 treatment failure following a standard three-day course of DHA-PPQ. Treatment failure rates
308 increased from 25% in 2010 to 46% in 2014^{11, 29, 74, 75, 76, 77} in western Cambodia, suggesting
309 that DHA-PPQ is becoming ineffective within only a few years of its nationwide deployment.
310 The high treatment failure rates suggest that parasites with reduced susceptibility to both

311 artemisinin and piperaquine are now prevalent in western/northern Cambodia. Clinical failures
312 of DHA-PPQ treatment have been linked to the emergence of PPQ resistance, but no strong
313 correlation has been found between clinical outcome (recrudescence two to four weeks after
314 the initial treatment) and susceptibility to PPQ *in vitro*, as assessed by the determination of
315 IC₅₀ for pre-treatment (day 0) isolates⁷⁷. Piperaquine resistance remains poorly characterized
316 and there is currently no robust evidence of parasite-dependent resistance to this drug
317 (reliable *in vitro* phenotype and/or validated genetic molecular marker).

318 ***In vitro detection of piperaquine resistance: Piperaquine Survival Assay (PSA)***. Several
319 *in vitro* studies conducted in Cambodia, have reported an increase over time in the geometric
320 mean IC₅₀ of PPQ in western Cambodia or a significantly higher geometric mean IC₅₀ of PPQ
321 for isolates from patients with recrudescent infections than for those from cured patients^{11, 74,}
322 ⁷⁶. However, no direct association between a high IC₅₀ or 90% inhibitory concentration (IC₉₀)
323 of PPQ for isolates before treatment and the failure of dihydroartemisinin-piperaquine
324 treatment has ever been demonstrated. In all published studies, the range of IC₅₀ values for
325 PPQ in patients with recrudescent infections overlapped substantially with those in cured
326 patients, suggesting that the classical *in vitro* test is not sufficiently reliable for the
327 identification of PPQ-resistant parasites and would therefore probably detect the elimination,
328 in a given area, of the parasites most sensitive to piperaquine^{11, 75}. It has been recently shown
329 that the poor performance of the standard isotopic assays for detecting piperaquine resistance
330 was associated with a relatively high frequency of non-interpretable curves in assays on
331 piperaquine-resistant isolates. Most of the isolates collected from patients with recrudescent
332 infection had non-interpretable IC₅₀ curves, whereas all yielded conventional response curves
333 for the other drugs tested. These anomalous curves presented a paradoxical increase in [³H]-
334 hypoxanthine incorporation at piperaquine concentrations above 100-200 nM, the
335 physiological concentration of piperaquine observed in the blood of patients treated with a
336 standard three-day course of dihydroartemisinin-piperaquine⁷⁷. To overcome the limitations
337 of current assays and provide a more robust assessment of piperaquine resistance, a new *in*

338 *vitro* assay (named ‘Piperaquine Survival Assay’, PSA) based on the same principle as the RSA
339 was developed⁷⁷. This assay involves exposing tightly synchronized culture-adapted parasites
340 (*in vitro* format) or parasites directly collected from patients (*ex vivo* format) to a
341 pharmacologically relevant dose of piperaquine (200 nM for 48 hours), to mimic *in vivo*
342 conditions. The *in vitro* PSA was found to discriminate efficiently between piperaquine-
343 resistant and sensitive parasites, with resistance defined as a survival rate in the PSA \geq 10%
344 (defined as the cut-off for resistance on the basis of results for the isolates of patients with
345 recrudescent infection). Patients harbouring parasites with K13 mutants with an *ex-vivo*
346 survival rate in the PSA \geq 10% had a 32 times higher risk of having recrudescent infection. The
347 use of the PSA (in absence of reliable molecular marker) together with K13 genotyping was
348 found to be a suitable method for monitoring resistance to both artemisinin and piperaquine,
349 in areas in which DHA-PPQ is currently the recommended first-line treatment and may be a
350 reliable platform for identifying molecular markers associated with piperaquine resistance and
351 for evaluating alternative drugs effective against piperaquine-resistant parasites.

352 **Toward a molecular marker for piperaquine resistance.** Studies conducted in Cambodia
353 and Thailand with the aim of characterizing molecular signatures associated with piperaquine
354 resistance have found no association between the *in vitro* PPQ susceptibility of *P. falciparum*
355 isolates and copy number variations for the PF3D7_0520500 and PF3D7_0520900 genes
356 located on chromosome 5 previously identified by Eastman *et al*^{11, 78, 79}. Spring *et al*. recently
357 reported a significant correlation between DHA-PPQ treatment failure and the presence of a
358 triple-mutant haplotype for the propeller domain of the K13 gene and for positions 1718319
359 and 688956 on chromosomes 13 and 10, respectively (MAL13: 1718319 and MAL10: 688956)
360 previously reported to be associated with delayed parasite clearance in genome-wide
361 association studies^{29, 35, 76}. Patients harboring triple-mutant parasites had a 5.4 times higher
362 risk of treatment failure and a higher IC₅₀ for PPQ (33.6 nM for the triple mutant *versus* 24 nM
363 for other parasites). However, rather than being directly associated with piperaquine resistance
364 (no causal impact and no biological rationale), these markers may be associated with a

365 particular artemisinin genetic background favoring the acquisition of resistance to
366 piperaquine⁸⁰.

367 However, several groups with different *in vitro* assays showed that parasites with single-copy
368 *Pfmdr-1* (and consequently a low IC₅₀ value for mefloquine) were strongly linked to
369 piperaquine resistance^{11, 76, 77}. For instance, it has been observed that all *in vitro* piperaquine-
370 resistant isolates had a single *Pfmdr-1* copy but the converse was not true, as not all isolates
371 with a single copy of *Pfmdr-1* were *in vitro* piperaquine-resistant. This finding strongly
372 supports recent recommendations for the use of artesunate plus mefloquine as an alternative
373 first-line treatment in provinces in which DHA-PPQ is failing^{11, 76, 77}. However, further
374 investigations are required to determine whether the elimination of parasites with multiple
375 copies of *Pfmdr-1* has resulted from the removal of mefloquine pressure or the selective
376 pressure exerted by piperaquine. This last finding also suggests that PfMDR1, a pump involved
377 in the intraparasitic influx/efflux of metabolites or drugs, may play a significant role in parasite
378 susceptibility to PPQ. For instance, PfMDR1 may play a significant role in the influx of PPQ into
379 the digestive vacuole, in which PPQ acts on its target to kill parasites. Nonetheless, some
380 parasites sensitive to piperaquine harbour a single copy of *Pfmdr1*, highlighting the need to
381 find a specific molecular marker.

382 Several studies have also observed that specific mutations of the *Pfcrt* gene were significantly
383 associated with piperaquine resistance^{77, 78, 81}. For instance, isolates containing parasites with
384 a variant of the Dd2 *Pfcrt* allele carrying either 97Y, 343L or 353V have higher median PSA
385 survival rates than those harbouring the Dd2 allele⁷⁷. In French Guiana, Pelleau *et al.* also found
386 that the acquisition of the C350R substitution in the *Pfcrt* gene decreased susceptibility to
387 piperaquine, suggesting that piperaquine pressure drove the expansion of this allele⁸¹. As the
388 353V mutation (frequently observed in Cambodia) is located close to 350R, further
389 investigations, such as genome editing, should be carried out to assess the role of *Pfcrt*
390 mutations in the genetic background of Cambodian parasites.

391 **Perspectives.** The development of the PSA as an efficient screening tool for PPQ-resistant
392 parasites is a major advance that may facilitate the identification of a specific molecular
393 marker. Molecular signatures (copy number variation and single-nucleotide polymorphism)
394 associated with *in vitro* resistance to piperaquine are being investigated by the whole-exome
395 sequencing of piperaquine-resistant (PSA survival rates \geq 10%) and PPQ-susceptible (PSA
396 survival rates< 10%) parasites. In parallel, we repeatedly exposed parasites to 200 nM PPQ,
397 over increasingly long exposure times (from 48 h to 96 h), over a period of one year
398 (unpublished work). This resulted in the generation of a Cambodian piperaquine-resistant
399 culture-adapted parasite (PSA survival rate of 21%). The survival rates in the PSA of the initial
400 parasite and its sibling clone cultured under the same conditions but without the drug were
401 estimated at 3% and 0.7%, respectively. The differences between the three exomes is analyzed,
402 and the results obtained should facilitate accurate definition of the molecular signatures
403 associated with piperaquine resistance, while this approach has already been successfully used
404 to identify mutations in the propeller domain of the *K13* gene as a major determinant of
405 artemisinin resistance.

406

407 **Concluding remarks**

408 Assessments of antimalarial drug efficacy include clinical drug efficacy studies, *in vitro*
409 assessments of drug sensitivity and molecular markers (Figure 3). Drug efficacy studies (clinical
410 phenotype) remains the gold standard to evaluate the efficacy of antimalarial drugs. Clinical
411 studies investigate both the parasite clearance time in the first three days of treatment to
412 detect artemisinin resistance and the treatment failure rate (evaluated during the follow-up
413 usually until day 42 or day 63 according to the half-life of the partner drugs) to detect partner
414 drug resistance. *In vitro* phenotype assessing the sensitivity of *P. falciparum* malaria parasites
415 to antimalarial drugs is also frequently used to complement data from clinical studies. These
416 assays contribute to detect early signs of emergence of drug resistance. *In vitro* assays are also

417 useful for drug screening and for validating candidate molecular markers associated with drug
418 resistance. Indeed, data obtained from *in vitro* assay are directly associated to the parasite
419 drug susceptibility and contrarily to *in vivo* phenotypes, are not affected by host factors, such
420 as acquired immunity, bioavailability or pharmacokinetics of antimalarial drugs. Classical *in*
421 *vitro* assays (assessing the 50% inhibitory concentration - IC₅₀ - values) have limited sensitivity
422 for detecting resistant parasites to artemisinin and piperaquine and the design of such assays
423 needs to be adapted according to the pharmacokinetic/pharmacology profiles of the tested
424 drugs. The RSA and the PSA are excellent examples. Finally, validated molecular markers
425 associated to antimalarial drug resistance are relevant to detect and monitor in real-time the
426 geospatial distribution of resistant parasites. They are useful to anticipate risks associated with
427 the emergence and the spread of antimalarial parasite resistance and to inform policy makers.
428 The discovery of the molecular signatures associated to antimalarial drug resistance is
429 challenging. However, at present, next generation sequencing of well characterized parasites
430 which allow to detect Single Nucleotide Polymorphisms (SNPs) and Copy Number Variations
431 (CNVs) differences between sensitive and resistant parasites greatly facilitate the genomic
432 investigations (Figure 3).

433 Resistance to both artemisinin and piperaquine is now prevalent in Cambodia, as
434 demonstrated by the worrying increase in the number of cases of DHA-PPQ treatment failure.
435 The decrease in ACT efficacy has resulted from adaptation of the parasite to both artemisinin
436 derivatives and the partner drug. Our understanding of artemisinin resistance has progressed
437 considerably, due, in particular, to the discovery of K13 polymorphisms. Evaluations of the
438 global distribution of artemisinin resistance, with the screening of thousands of blood samples
439 for the detection of K13 mutations, are underway. However, greater efforts are required
440 concerning piperaquine resistance. There is an urgent need to characterize piperaquine
441 resistance in more detail and to identify a reliable and specific molecular marker of this
442 resistance, as this drug is a potential partner drug candidate for inclusion in combinations with

443 the next generation of antimalarial drugs, such as ozonides, spiroindolones, and
444 imidozolopiperazines⁸².

445 The management of falciparum malaria cases in Cambodia remains challenging. New
446 strategies (even short-term approaches) using existing antimalarial drugs are required. These
447 approaches may include a rotation of different ACTs (DHA-piperaquine and artesunate-
448 mefloquine), which could be effective due to the contrasting susceptibility profiles of
449 piperaquine- and mefloquine-resistant parasites. However, such strategies would be expected
450 to fail in the long term, with the emergence of parasites resistant to both PPQ and MQ. This
451 strategy is currently implemented in several provinces in Cambodia in which high failure rates
452 have been recorded for DHA-PPQ treatment. Another option is extending the course of
453 treatment to five or seven days, but it may be difficult to ensure compliance with a longer
454 course of treatment. Finally, another alternative approach would involve combining
455 artemisinin derivatives with two slowly eliminated partner drugs in a three-day triple ACT, in
456 a strategy similar to those already used for HIV and tuberculosis. This approach could involve
457 the use of artemether-lumefantrine-amodiaquine and DHA-piperaquine-mefloquine
458 combinations, which are currently under evaluation for the treatment of uncomplicated
459 falciparum malaria in areas of resistance to both artemisinin and the partner drug in
460 conventional ACT.

461

462 **Acknowledgments**

463 We thank all the patients enrolled in the studies, the health center staff and the staff from the
464 National Center for Parasitology, Entomology and Malaria Control in Cambodia for their
465 support. We thank the staff of the Institut Pasteur in Cambodia and those from the Malaria
466 Molecular Epidemiology Unit, especially Nimol Khim, Nimol Kloeung, Sopheavkatey Ke and
467 Anais Domergue. We also want to thank Pascal Ringwald for providing in-depth data of ACT

468 efficacy studies conducted in Cambodia. We also thank the Division Internationale of the
469 Pasteur Institute (Marc Jouan and Eliane Coeffier).

470

471 **Financial Support**

472 This study was supported by the Institut Pasteur in Cambodia, the Institut Pasteur, Paris
473 through a Programme Transversal de Recherche grant (PTR 2015 – 535) and the KARMA
474 project and the 5% initiative (Vers l'élimination du paludisme ou comment agir efficacement
475 contre la transmission des parasites du paludisme ? Prochains défis à relever pour les pays du
476 Sud-est asiatique). Valentine Duru is supported by a doctoral fellowship from the International
477 Division, Institut Pasteur.

478

479 **Disclosures**

480 All authors have seen and approved the submitted manuscript and have no real or perceived
481 conflict of interest to declare.

482

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499 **Figure legends**

500

501 **Figure 1:** Timeline between the introduction of the main antimalarial drugs and the first case
502 of emergence of resistance.

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532 resistance to artemisinin and piperaquine in western Cambodia: dihydroartemisinin-piperaquine
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536

537 **Figure 2:** Spatial distribution and evolution of the efficacy of artesunate-mefloquine and
538 dihydroartemisinin-piperaquine 3 day-standard course in different sites in Cambodia
539 between 2001 and 2014. Details are provided in Table 1.

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542

543 **Figure 3:** Main tools and strategy required for the detection of the molecular signatures
544 associated with antimalarial drug resistance.

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551 **Table**

552

553 **Table 1:** Detailed data of the therapeutic efficacy studies performed in different sites in
 554 Cambodia between 2001 and 2015 to evaluate the efficacy of 3 day-standard course of ACTs
 555 (artesunate-mefloquine, dihydroartemisinin-piperaquine, artesunate-pyronaridine and
 556 artemether-lumefantrine).

557

558

Study sites (Province)	Start Year	End Year	Follow up (days)	Antimalarial drug regimen	ACPR (%)	Reference
Battambang	2001	2001	28	AS-MQ	96.0	62
	2003	2003	28	AS-MQ	92.3	62
	2005	2005	42	AS-MQ	94.1	#
	2007	2007	42	AS-MQ	95.4	#
	2012	2013	42	DHA-PPQ	67.5	77
	2014	2014	42	ATS/PYRO	100.0	#
Kampong Speu	2003	2003	28	AS-MQ	96.7	62
	2010	2010	42	AS-MQ	88.9	74
	2012	2013	42	DHA-PPQ	86.4	77
Kampong Thom	2012	2012	42	DHA-PPQ	100.0	#
	2012	2013	42	DHA-PPQ	100.0	77
Kampot	2006	2008	42	AS-MQ	81.2	83
	2013	2014	42	DHA-PPQ	94.1	#
Kratie	2001	2001	28	AS-MQ	100.0	62
	2003	2003	28	AS-MQ	100.0	62
	2006	2006	42	AS-MQ	97.5	#
	2011	2011	42	DHA-PPQ	96.4	#
	2011	2012	42	DHA-PPQ	83.3	77
	2013	2013	42	DHA-PPQ	100.0	#
Mondulkiri	2014	2015	42	DHA-PPQ	90.0	#
Odder Meanchey	2003	2003	28	AS-MQ	97.8	62
	2012	2013	42	DHA-PPQ	62.5	75
	2012	2014	42	DHA-PPQ	51.1	76
Pailin	2002	2002	28	AS-MQ	85.7	62
	2004	2004	28	AS-MQ	90.1	62
	2004	2004	42	AS-MQ	79.3	62
	2007	2008	42	AS-MQ	95.0	10
	2007	2008	42	AS-MQ	100.0	72
	2007	2008	42	ATS/PYRO	89.8	72
	2008	2009	42	DHA-PPQ	89.4	74
	2009	2010	42	DHA-PPQ	91.9	74
	2010	2011	42	DHA-PPQ	75.0	74
	2011	2011	42	AS-MQ	100.0	#
Preah Vihear	2014	2015	42	ATS/PYRO	82.0	84
	2002	2002	28	AS-MQ	96.6	62
	2004	2004	28	AS-MQ	100.0	62

	2006	2006	42	AS-MQ	98.6	#
	2007	2007	42	AS-MQ	100.0	#
	2009	2009	42	DHA-PPQ	100.0	74
	2009	2009	42	AS-MQ	100.0	#
	2011	2011	42	DHA-PPQ	96.6	#
	2011	2012	42	DHA-PPQ	93.4	77
	2012	2013	63	DHA-PPQ	84.6	11
	2013	2014	42	DHA-PPQ	93.7	#
Pursat	2002	2002	28	AS-MQ	94.0	62
	2004	2004	28	AS-MQ	92.6	62
	2005	2005	42	AS-MQ	87.5	#
	2005	2005	28	A/L	82.4	77
	2005	2005	28	DHA-PPQ	98.2	77
	2007	2007	42	AS-MQ	87.9	#
	2008	2008	42	DHA-PPQ	98.7	74
	2010	2010	42	DHA-PPQ	89.3	74
	2011	2011	42	DHA-PPQ	83.3	#
	2011	2012	42	DHA-PPQ	79.5	77
	2012	2013	63	DHA-PPQ	63.2	11
	2012	2013	42	DHA-PPQ	97.5	77
	2014	2015	42	ATS/PYRO	89.8	84
Rattanakiri	2002	2002	28	AS-MQ	100.0	62
	2004	2004	28	AS-MQ	100.0	62
	2006	2006	42	AS-MQ	96.2	#
	2009	2010	42	DHA-PPQ	100.0	74
	2010	2010	28	A/L	95.0	#
	2010	2010	42	DHA-PPQ	100.0	74
	2010	2010	42	DHA-PPQ	100.0	#
	2012	2013	63	DHA-PPQ	98.4	11
	2013	2013	42	DHA-PPQ	98.3	#
	2014	2014	42	DHA-PPQ	67.6	77
Siem Reap	2014	2015	42	DHA-PPQ	37.5	#
Stung Treng	2014	2015	42	DHA-PPQ	65.6	#

559 ACPR: adequate clinical and parasitological response (proportion, %); Follow up: the last day of
 560 follow-up according to the WHO protocol used (28, 42 or 63 days); AS-MQ: artesunate + mefloquine;
 561 DHA-PPQ: dihydroartemisinin + piperaquine; ATS/PYRO: artesunate + pyronaridine; A/L:
 562 artemether + lumefantrine; #: National Center for Parasitology Entomology and Malaria
 563 Control (CNM) unpublished reports.

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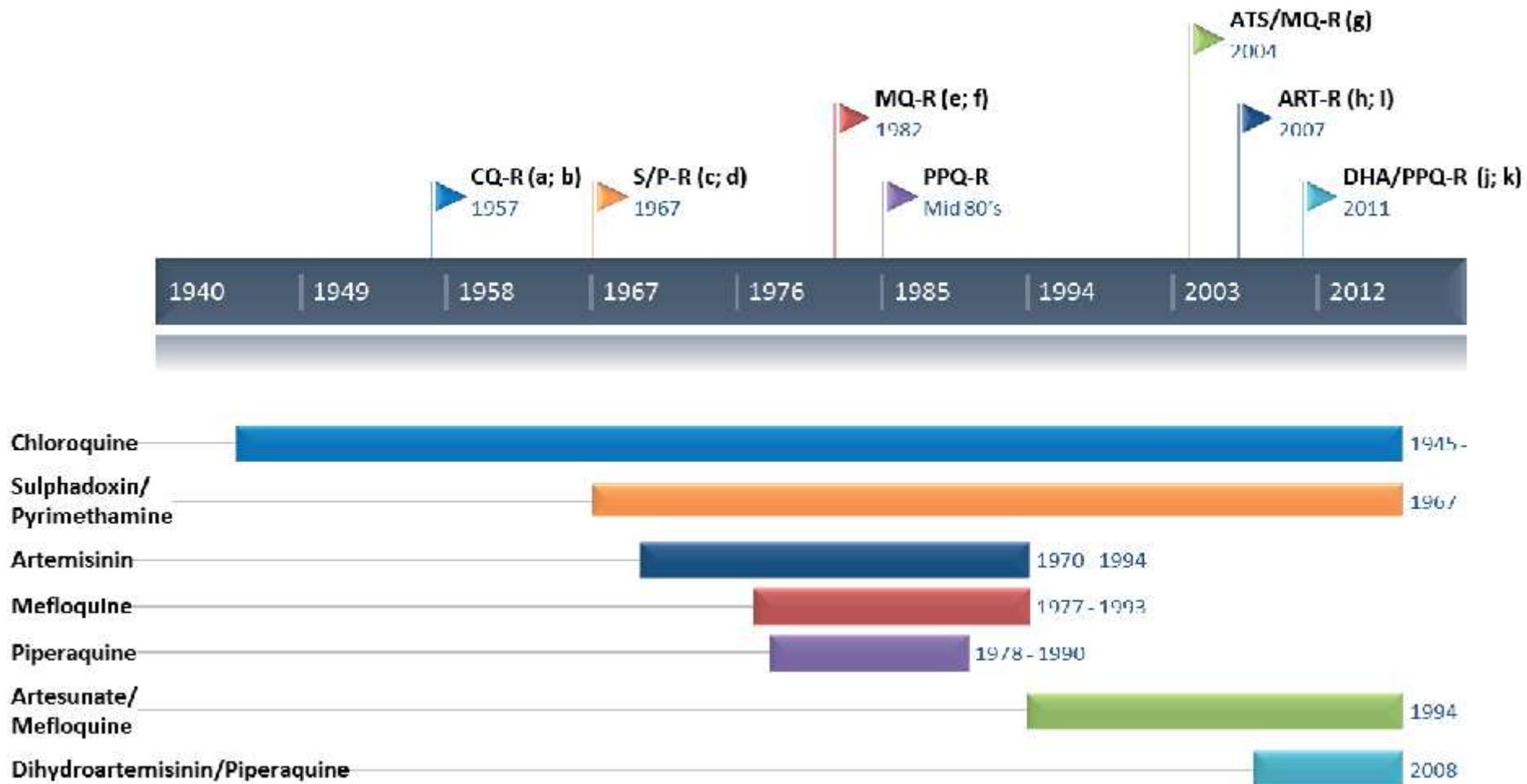
Figure 1

Figure 2

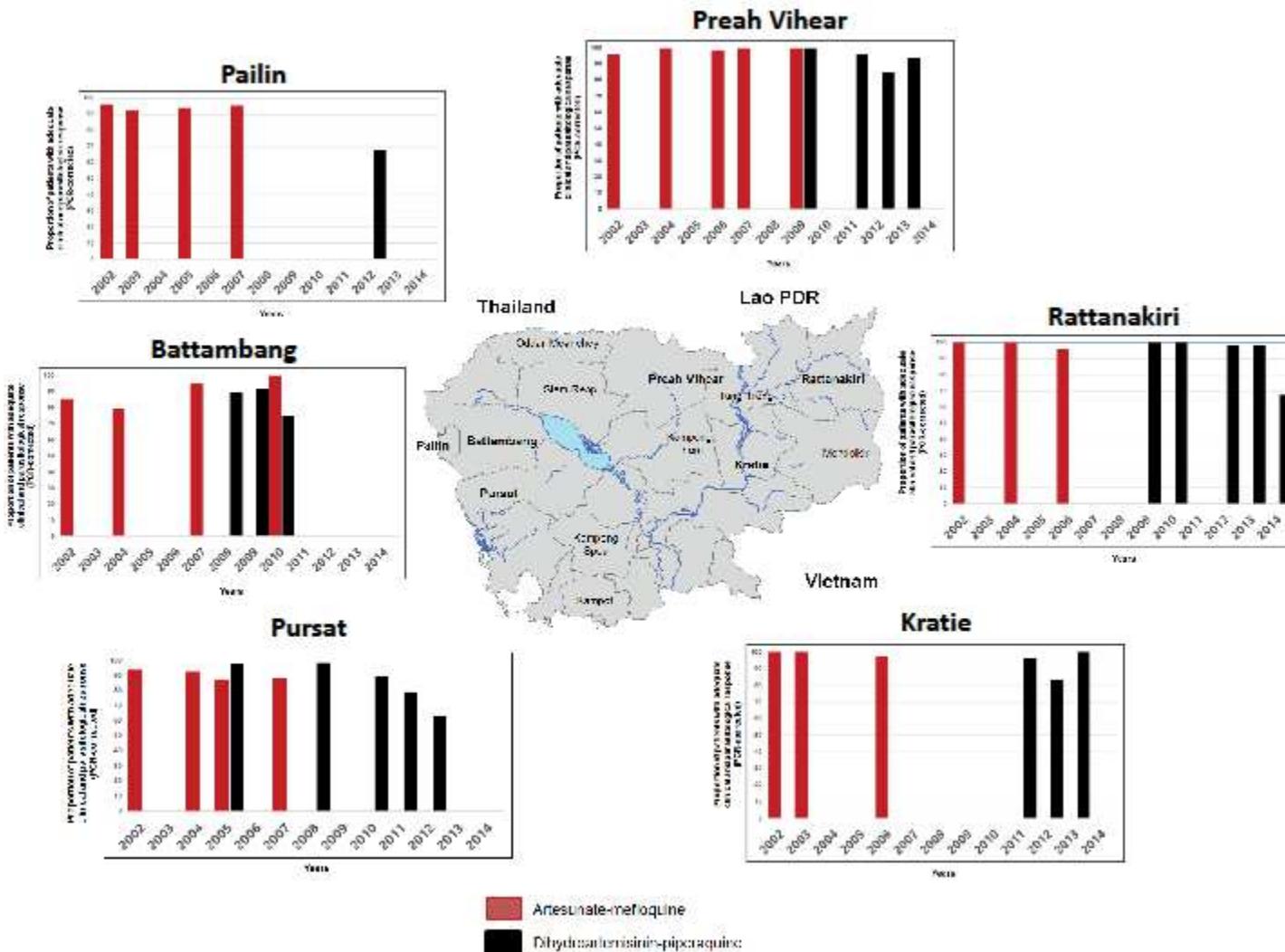
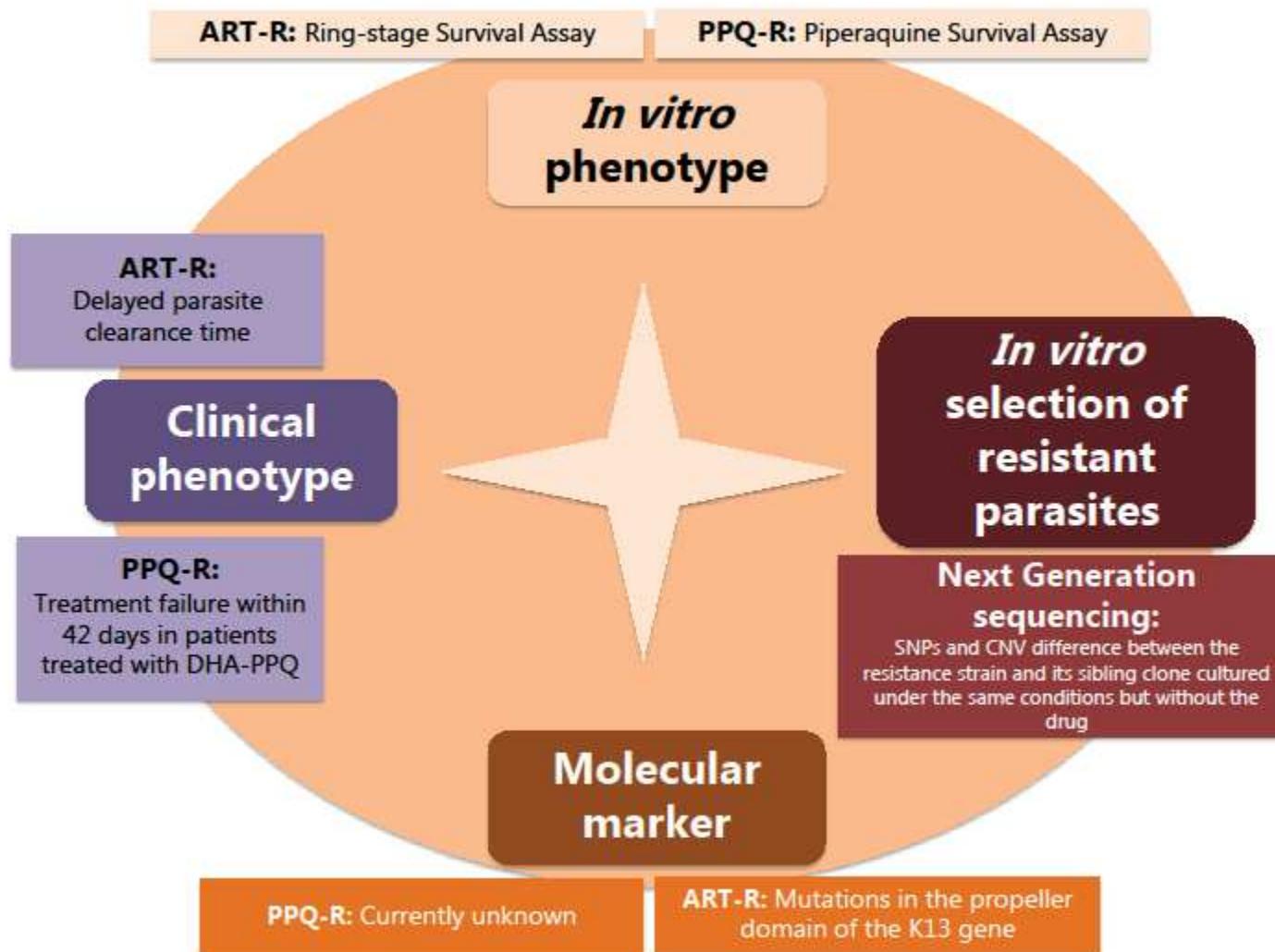


Figure 3

Conclusion

La résistance à la fois à l'artémisinine et à la pipéraquine est aujourd’hui prévalente au Cambodge, comme en témoigne l’augmentation inquiétante du nombre d’échecs de traitement à la DHA-PPQ. Le déclin de l’efficacité de la DHA-PPQ est le résultat direct de l’adaptation des parasites aux dérivés d’ART et à la drogue partenaire.

L’évaluation de l’efficacité des molécules antipaludiques passe par des études d’efficacité clinique, des analyses *in vitro* de sensibilité à la drogue, et l’utilisation de marqueurs moléculaires (Fig. 11). Les études cliniques menées à intervalles réguliers dans des sites sentinelles permettent la détection précoce de la perte d’efficacité d’un antipaludique, mais exigent des moyens logistiques et financiers importants qui les rendent peu adaptées à une surveillance à grande échelle. De plus, l’efficacité d’un traitement dépend de plusieurs facteurs, tels que l’administration dudit traitement, l’immunité du patient, la pharmacodynamique, etc... Les tests phénotypiques tels que les tests d’ IC_{50} ou les *Survival Assays* (RSA ou PSA) permettent de s’affranchir de ces facteurs confondants et d’obtenir un résultat dépendant uniquement de la résistance intrinsèque du parasite, ce qui facilite la recherche de marqueurs moléculaires. Ils peuvent également s’avérer utiles pour tester de nouvelles molécules antipaludiques. Toutefois, les tests phénotypiques nécessitent une fois encore des moyens logistiques avancés (matériels et consommables de culture cellulaire, incubateur, conservation des échantillons pour envoi ou stockage, formation de personnel compétent, etc...) qui ne sont pas à la portée de tous les laboratoires. Les marqueurs moléculaires sont donc essentiels pour une surveillance à grande échelle efficace et à moindre de coût (une goutte de sang séché est facile à prélever et à stocker, et suffisante pour une analyse moléculaire), et peuvent également apporter des informations précieuses quant aux mécanismes impliqués dans la résistance lorsque leur causalité est établie.

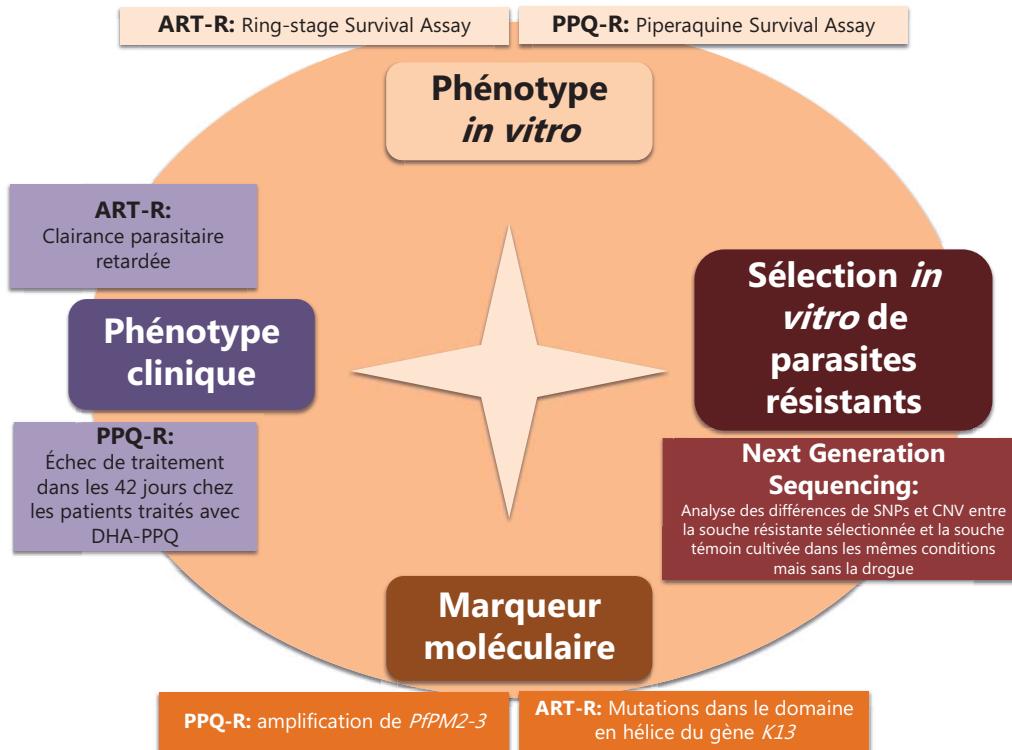


Figure 11. Outils principaux pour l'étude de la résistance aux molécules antipaludiques

Nos connaissances sur la résistance à l'ART ont beaucoup progressé, particulièrement grâce à la découverte des polymorphismes du gène *K13* comme marqueur moléculaire. Ce marqueur a permis à notre équipe et à d'autres^{52, 93-105} d'étudier la distribution de la résistance à l'ART à l'échelle mondiale, et de montrer que cette dernière était pour le moment confinée en Asie du Sud-Est. La preuve de la causalité du gène *K13* dans la résistance à l'ART a également ouvert la voie à l'étude des mécanismes impliqués¹⁰⁶, dont la compréhension peut être précieuse dans un contexte de développement de nouvelles molécules antipaludiques.

Il était tout aussi important de parvenir à caractériser au mieux la résistance à la PPQ, notamment parce que cette molécule est envisagée comme potentielle drogue partenaire de molécules antipaludiques de nouvelles générations¹⁰⁷: il est donc crucial de pouvoir s'assurer de son efficacité dans les régions où ces nouvelles combinaisons pourraient être utilisées. Nous avons décidé d'aborder le problème de la résistance à la PPQ en suivant la

même méthodologie que celle ayant aboutie à la découverte d'un marqueur moléculaire pour la résistance à l'ART ; cette méthodologie s'appuie sur plusieurs points :

- avoir accès à des parasites résistants ; notre situation au Cambodge, en plein cœur du problème, était pour cela idéale ;
- être capable de caractériser les-dits parasites résistants au moyen d'un test phénotypique fiable ; les tests standards d' IC_{50} s'étant montré peu concluants pour la PPQ, nous avons développé le PSA qui repose sur les mêmes principes que le RSA (dose et temps d'exposition pharmacologiquement pertinents) ;
- rechercher les signatures moléculaires associées à la résistance à partir soit d'une lignée sélectionnée (F32-ART dans le cas de l'ART et des polymorphismes *K13*) soit de 2 groupes bien caractérisés (par le PSA dans le cas de la PPQ et du CNV de *PfPM2-3*) ; les nouvelles technologies de Next Generation Sequencing offrent la possibilité de détecter à la fois les SNPs et les CNVs, facilitant grandement les analyses génomiques.

Cette méthodologie a de nouveau fait ses preuves puisqu'elle nous a permis d'identifier un marqueur moléculaire candidat pour la résistance à la PPQ, les CNVs de *PfPM2-3*. Des études supplémentaires doivent encore être menées afin de valider ce marqueur moléculaire et de pouvoir envisager une cartographie mondiale de la résistance à la PPQ, à l'image du projet KARMA.

Dans le cas de la résistance à l'ART comme dans celui de la résistance à la PPQ, c'est la première fois qu'un marqueur moléculaire est identifié avant diffusion de la résistance en Afrique. C'est un avantage capital puisqu'une surveillance à grande échelle efficacement mise en place permettra d'adapter rapidement les programmes nationaux de lutte contre le paludisme en cas de détection précoce de résistance, et d'éviter ainsi des catastrophes

sanitaires comme celle provoquée par la résistance à la chloroquine. Dans l'immédiat, les stratégies pouvant être mise en place en cas de résistance confirmée incluent une rotation entre différents TCAs (comme c'est le cas au Cambodge où l'ATS-MQ remplace la DHA-PPQ dans les zones où cette dernière n'est plus efficace), un prolongement du traitement, ou encore une trithérapie combinant un dérivé d'artémisinine et deux drogues partenaires, en attendant la mise sur le marché de molécules antipaludiques de nouvelle génération¹⁰⁷.

Épilogue

« Au bon endroit, au bon moment » : ces mots pourraient assez bien résumer le déroulement de ma thèse. Je suis arrivée au Cambodge, dans le laboratoire du Dr Didier Ménard, quelques mois après que le développement du test RSA, alors que la chasse au marqueur moléculaire pour la résistance à l'artémisinine battait son plein. Trois mois plus tard, l'équipe (en collaboration avec celle d'Odile Mercereau-Puijalon et Frédéric Ariey à Paris et celle de Françoise Benoit-Vical à Toulouse) avait identifié les polymorphismes du gène *K13* comme marqueur moléculaire candidat prometteur. J'avais pris le train en marche, commençais tout juste à pouvoir maintenir une culture de *Plasmodium falciparum* sans la contaminer et réaliser un test RSA de A à Z, et je me retrouvais face au « Graal ». J'ai eu la chance de pouvoir participer à la validation de ce marqueur moléculaire et ainsi de me retrouver co-auteur d'une publication dans *Nature*. S'en est suivi une collaboration tout aussi fructueuse avec l'équipe de David Fidock à New York durant laquelle, à grand renfort de nucléases à doigt de zinc et de RSA, nous avons pu confirmer le rôle majeur du gène *K13* dans la résistance à l'artémisinine. Et publier un article dans *Science*. Enfin, l'imposant projet KARMA, avec ses 14 000 échantillons et ses nombreux collaborateurs, s'est conclu par une publication dans le prestigieux *New England Journal of Medicine*. Bien que n'ayant pas été à l'initiative de ces projets, j'ai beaucoup apprécié pouvoir y prendre part et me rendre compte de l'importance de la collaboration dans le domaine de la recherche et de la santé publique. De plus, même si ma contribution à ces travaux fut principalement technique (culture cellulaire et tests *in vitro*), l'expérience aura été très formatrice pour la suite de ma thèse.

Ma première année de thèse a donc été centrée sur la résistance à l'artémisinine. Il y aurait eu, et il y a toujours, encore beaucoup de choses à faire sur ce sujet. Cependant, notre position en première ligne de l'émergence de la résistance à la pipéraquine au Cambodge nous a amené à nous concentrer sur ce nouveau problème, ce qui m'a réellement permis de prendre mon envol. Le développement du test du PSA et la recherche d'un marqueur

moléculaire pour la résistance à la pipéraquine sont des projets dans lesquels j'ai été impliquée dès leur genèse, et je suis fière d'avoir pu les voir aboutir. Parallèlement à ces travaux, j'ai également travaillé durant 18 mois à la sélection d'une souche résistante à la pipéraquine, et d'une autre, sa jumelle, résistante à la méfloquine, dans l'optique d'éclaircir la dichotomie qui semble exister entre ces deux types de résistance. Les analyses des exomes de ces deux souches, de la souche initiale et de la souche contrôle (cultivée dans les mêmes conditions mais sans drogue) sont en cours, mais les premiers résultats semblent prometteurs et pourraient faire l'objet d'une publication ultérieure.

En définitive, j'ai énormément apprécié l'opportunité de pouvoir réaliser ma thèse sur un problème de santé publique majeur, et ce à l'épicentre même de l'émergence de la résistance aux antipaludiques. Bien que j'aie pu m'essayer à une bonne partie des techniques mises en œuvre dans notre laboratoire (culture cellulaire, cytomètre, ELISA, PCR, Western Blot...), c'est définitivement dans le domaine de la biologie cellulaire que je me sens le plus à l'aise, et je préfère manipuler du vivant que de l'ADN... Mais je n'exclue pas de devoir un jour aiguiser mes compétences en biologie moléculaire lorsque cela sera nécessaire.

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Annexes

Article annexe a

Artemisinin-Resistant Plasmodium falciparum K13 Mutant Alleles, Thailand-Myanmar Border.

Boullé M, Witkowski B, Duru V, Sriprawat K, Nair SK, McDew-White M, Anderson TJ, Phyothiri AP, Menard D, Nosten F.

Emerg Infect Dis. 2016 Aug;22(8):1503-5. doi: 10.3201/eid2208.160004.

latter being more aggressive. In the Greater Metropolitan Area and Costa Rica in general, free-ranging dogs are common, including at playgrounds and school yards, sites also vulnerable to nocturnal visits by raccoons. Dogs can have patent *B. procyonis* parasite infections and can play a role in transmission of the parasite from raccoons to humans.

In Costa Rica, cases of larva migrans have been reported. The Unidad de Investigación y Análisis, Registros y Estadísticas de Salud at the National Children's Hospital, San José, Costa Rica, reported 135 cases of larva migrans ocularis and 21 cases of visceral larva migrans caused by nonspecifically identified ascarids during 2005–2014 (unpub. data). However, these diagnoses were based on IgG serologic testing results (Martinez J., National Children's Hospital; pers. comm., 2015), which do not identify ascarid species. Western blot testing would improve accuracy (10).

The eco-epidemiology of *B. procyonis* parasites in tropical settings is relevant to public health because it might play a yet-unrecognized role in larva migrans pathology, which can be severe. Increased contact between raccoons and humans also warrants further investigation to improve understanding and minimize zoonotic risk.

This work was funded by a grant from the Fondo Institucional de Desarrollo Académico-2013-Universidad Nacional-Universidad de Costa Rica, Fondo del Sistema-Consejo Nacional de Rectores (ACUERDO-VI-167-2013).

The study was approved by Research Ethics Board permits (FCSA-EMV-CBA-007-2013); the Universidad Nacional de Costa Rica, Universidad de Costa Rica Institutional Committee for the Use and Care of Laboratory Animals (CICUA-130-13), and the Institutional Review Board of MINAE (ACCVC-OH-512).

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Artemisinin-Resistant *Plasmodium falciparum* K13 Mutant Alleles, Thailand–Myanmar Border

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DOI: <http://dx.doi.org/10.3201/eid2208.160004>

To the Editor: Artemisinin resistance (ART-R) in *Plasmodium falciparum* phenotypes may have evolved independently in various areas of the Greater Mekong Subregion (1,2), prompting the World Health Organization to change its regional policy from containment to elimination (3). Risks associated with ART-R include compromised use of artemisinin combined therapy, partner drug resistance selection, total ART-R resistance, and geographic extension

¹These authors contributed equally to this article.

to other malaria-endemic regions (2,3). Characterization of ART-R in each setting and rapid update of listed phenotypes classified as in vitro resistant to this antimalarial drug are needed.

Detected in western Cambodia in 2008, ART-R has been observed in neighboring countries, notably at the Thailand–Myanmar border (4,5). Resistance is partial and manifests by an increased parasite clearance half-life (PCHL) of >5 hours in patients receiving artemisinin monotherapy or artemisinin combined therapy (6). In vitro, ART-R phenotype has been characterized by the ring-stage survival assay (RSA^{0–3h}, cutoff 1%) (7) and mutations in the propeller domain of a *kelch* gene on chromosome 13 (K13) (8,9). However, tremendous K13 variability in different genetic backgrounds requires confirmation of specific alleles as ART-R markers (2,3); even statistically significant clinical associations are rarely unequivocal (5–9).

On the Thailand–Myanmar border where ART-R has been documented (4), we investigated K13 mutations in clinical and in vitro phenotypes. Patients with nonsevere *P. falciparum* hyperparasitemia infections treated during 2011–2013 at the Shoklo Malaria Research Unit (Mae Sot, Thailand) were treated with artesunate, then combined artesunate/mefloquine (5). We retrospectively selected 33 case-patients on the basis of PCHL outcome to analyze a broad parasite clearance distribution with available cryopreserved isolates. Full written consent from all patients was obtained. PCHL was calculated on the basis of initial and repeated parasitemia measurement every 6 hours until undetectable asexual parasitemia (6) was achieved. Venous blood samples were cryopreserved before drug administration (day 0).

Short-term, culture-adapted parasites (3% hematocrit; RPMI-1640 supplemented with 10% human serum, 0.05 mg/mL hypoxanthine, 2 mg/mL sodium bicarbonate, 2 mg/mL glucose, 0.04 mg/mL gentamicin, 0.3 mg/mL L-glutamine in a 37°C candle-jar atmosphere) were split for blinded RSA^{0–3h} and K13 genotyping. We performed RSA^{0–3h} in duplicate by selecting early rings (0–3 h) in a combination of percoll gradient and sorbitol lysis, followed by a 6-h exposure to 700 nmol/L dihydroartemisinin (7). RSA survival rate was measured microscopically 66 hours after drug removal and calculated as the quotient of parasitemia upon DHA exposure over control parasitemia with dimethyl sulfoxide. Only 25 isolates that were successfully culture-adapted provided RSA survival rates.

After the phenotypical assays, the genotypes were obtained and K13 regions were amplified by using 3 primer sets: fragment 1 (1725380–1725680 bp, pos 211–302), F-tgaaaatggtagtgatt and R-atcgttccatgttcttct; fragment 2 (1725980–1726520 bp, pos 419–570), F-atcattgggttcaagg, R-ccaaaagatctaagtgaaag; and fragment 3 (1726400–1726940 bp, pos 545–707), F-ctgccattcattgtatct,

R-ggatatggcttctta) before sequencing (8). The 33 monoclonal isolates yielded clear K13 gene sequences. All except 4 isolates from patients who had PCHL >5 h had a single K13 mutant allele (19/23), and all isolates except 1 (G538V) from case-patients who had PCHL <5 h carried the K13 3D7 wild-type allele (9/10). PCHL was significantly different between K13 wild-type ($n = 13$, median 4.3 h) and mutant ($n = 20$, median = 7.2 h) alleles ($p < 0.01$ by Mann-Whitney U test). Among the 25 isolates successfully tested, RSA survival rates differed significantly between K13 wild-type ($n = 10$, median 0.5%) and mutant ($n = 15$, median 3.5%) alleles ($p < 0.001$ by Mann-Whitney U test). When PCHL was present <5 h, RSA survival rates ($n = 7$, median 0.5%) were significantly lower than when PCHL was >5 hours ($n = 18$, median 3.1%) ($p = 0.001$ by Mann-Whitney U test).

In detail (Figure), C580Y and N458Y mutants were consistently associated with PHCL >5h and RSA values >1%. The C580Y allele has been repeatedly confirmed as a molecular marker of ART-R (5,7–9). Previous reports have inconsistently associated the N458Y mutation with ART-R; 7 case-patients with PCHL >5 h were reported by Ashley et al. (5), and 1 artemisinin sensitive case was reported at the China–Myanmar border (10). Nevertheless, this mutation has not been confirmed in vitro (3). We confirmed the mutation in vitro, and in vivo, according to the World Health Organization definition (3), this K13 allele as a molecular marker of ART-R.

Conflicting data observed between PCHL and RSA values for 4 mutant alleles (E252Q, P441L, G538V, and

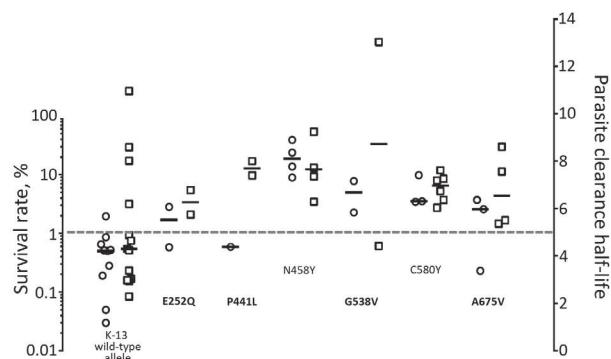


Figure. Distribution of parasite clearance half-lives ($n = 33$, squares) and Ring-stage survival assay survival rates ($n = 25$, circles) of *Plasmodium falciparum* isolates from patients on the Thailand–Myanmar border, determined on the basis of each K13 genotype. Mean survival rate of duplicate measures are showed for each isolate. Dashed line represents the cutoff value for parasite clearance half-life (artemisinin resistance >5 h) and RSA survival (artemisinin resistance >1%). K13 alleles N458Y and C580Y were consistently associated with parasite clearance half-life and survival rates above threshold. Bold text indicates K13 alleles with variable parasite clearance half-life and RSA associations. Horizontal bars represent median values for each K13 genotype. Survival rate for laboratory reference 3D7 strain was 0.2%.

A675V) require further targeted approaches to relate them to previous reports. In a study in which only PCHL were reported (5), the proportion of slowly clearing infections were 69%, 0%, 30%, and 61% for the P441L, E252Q, G538V, and A675V alleles, respectively. Discrepancies can result from confounding pharmacologic (drug level, partner drug), immunologic, and parasitologic (genetic background, parasitic stage at treatment initiation) factors.

RSA results and K13 genotypes were associated with delayed parasite clearance, emphasizing the pertinence of each method to define ART-R. In this area, N458Y is a marker of ART-R. To solve conflicts about specific mutations, more detailed characterization in vitro and in vivo is needed.

Acknowledgments

We thank all the patients and their parents or guardians.

The Shoklo Malaria Research Unit is part of the Mahidol-Oxford University Research Unit, funded by the Wellcome Trust of Great Britain. Laboratory work at Texas Biomedical Research Institute was conducted in facilities constructed with support from Research Facilities Improvement Program grant C06 RR013556 and R017515 from the National Center for Research Resources of the National Institutes of Health and was funded by National Institute of Allergy and Infectious Diseases Grant R37AI048071. This work was supported by grants from the Réseau International des Instituts Pasteur ACIP grant #A14-2012.

M.B., B.W., F.N., and D.M. contributed to the study design. M.B., B.W., and V.D. performed the in vitro assays. T.A., S.N., M.M-W., and K.S. performed the genetic polymorphism analyses. A.P.P. and F.N. coordinated and supervised the clinical studies. M.B., B.W., F.N. and D.M. analyzed the data and wrote the first draft of the manuscript. All authors contributed to the writing of the manuscript.

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Meningococcal Group W Disease in Infants and Potential Prevention by Vaccination

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DOI: <http://dx.doi.org/10.3201/eid2208.160128>

To the Editor: We recently reported that postvaccination serum samples from infants immunized with a novel, protein-based multicomponent meningococcal serogroup B (MenB) vaccine (Bexsero; GlaxoSmithKline Vaccines, Verona, Italy) have bactericidal activity against the hypervirulent meningococcal group W (MenW) strain belonging to the sequence type (ST) 11 clonal complex (1). Historically, MenW has been a rare cause of invasive meningococcal disease (IMD), accounting for <5% of confirmed cases in England and Wales (2). Since 2009, MenW cases caused by

Article annexe b

Plasmodium copy number variation scan: gene copy numbers evaluation in haploid genomes.

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Malar J. 2016 Apr 12;15:206. doi: 10.1186/s12936-016-1258-x.

RESEARCH

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Plasmodium copy number variation scan: gene copy numbers evaluation in haploid genomes

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Abstract

Background: In eukaryotic genomes, deletion or amplification rates have been estimated to be a thousand more frequent than single nucleotide variation. In *Plasmodium falciparum*, relatively few transcription factors have been identified, and the regulation of transcription is seemingly largely influenced by gene amplification events. Thus copy number variation (CNV) is a major mechanism enabling parasite genomes to adapt to new environmental changes.

Methods: Currently, the detection of CNVs is based on quantitative PCR (qPCR), which is significantly limited by the relatively small number of genes that can be analysed at any one time. Technological advances that facilitate whole-genome sequencing, such as next generation sequencing (NGS) enable deeper analyses of the genomic variation to be performed. Because the characteristics of *Plasmodium* CNVs need special consideration in algorithms and strategies for which classical CNV detection programs are not suited a dedicated algorithm to detect CNVs across the entire exome of *P. falciparum* was developed. This algorithm is based on a custom read depth strategy through NGS data and called PlasmoCNVScan.

Results: The analysis of CNV identification on three genes known to have different levels of amplification and which are located either in the nuclear, apicoplast or mitochondrial genomes is presented. The results are correlated with the qPCR experiments, usually used for identification of locus specific amplification/deletion.

Conclusions: This tool will facilitate the study of *P. falciparum* genomic adaptation in response to ecological changes: drug pressure, decreased transmission, reduction of the parasite population size (transition to pre-elimination endemic area).

Keywords: Malaria, Anti-malarial drug resistance, Copy number variation, Bioinformatics

Background

The burden of malaria has decreased by half over the last decade. This is a direct consequence of effective strategies mainly focused on vector control (long-lasting impregnated bed nets) and the management of suspect malaria cases (early diagnosis by rapid diagnostic tests and effective and prompt treatment with artemisinin-based combination therapy). As a consequence, a drastic

decrease in *Plasmodium falciparum* population biomass in many countries has been observed [1]. This new epidemiological situation has led to a change in the environment within which the parasite finds itself and will thus alter the selective pressures on parasite populations.

Natural evolution of malaria parasites generates an enormous amount of genetic diversity either linked with copy number variations (CNVs), or acquisition of new single nucleotide variations (SNVs) and their accumulation over time [2]. This allows parasites to acquire a high capacity of adaptation to the environmental shifts and develop anti-malarial drug resistance. Indeed, SNVs are known to be at the origin of resistance to anti-malarial

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drugs, such as chloroquine, sulfadoxine, pyrimethamine, atovaquone, artemisinin, and *mdr1* gene amplification is known to be at the origin of mefloquine resistance [3–5].

In eukaryotic genomes, SNP mutation rates occur at a rate of $\sim 10^{-8}$ per generation and deletion or amplification rates have been estimated to be in the order of $\sim 10^{-4}$ per generation [6, 7]. The number of *P. falciparum* parasites infecting an adult can be estimated from 5 to 50 billion parasites (10^3 – 10^4 parasites per μL of blood with a total of 5 l of blood). Because asexual replication occurs every 48 h, the erythrocytic stage of *P. falciparum*, therefore, appears to be a breeding ground for any selection pressure to act on parasite population. Although the regulation of gene expression in *P. falciparum* is still incompletely understood, relatively few transcription factors have been identified [8, 9] and the regulation of transcription is seemingly largely influenced by gene amplification events. Thus CNV is a major mechanism enabling parasite genomes to adapt to new environmental changes.

Currently, the detection of CNVs is based on quantitative PCR (qPCR), which is significantly limited by the relatively small number of genes that can be analyzed at any one time, and the fact that endogenous controls (e.g., housekeeping genes) can introduce bias into the results if not properly chosen [10]. Technological advances that facilitate whole-genome sequencing such as Next Generation Sequencing (NGS) enable deeper analyses of the genomic variation to be performed. Because the characteristics of *Plasmodium* CNVs need special consideration in algorithms and strategies for which classical CNV detection programs are not suited, a dedicated algorithm to detect CNVs across the entire exome of *P. falciparum* based on a custom read depth strategy through NGS data was developed. This algorithm was named PlasmoCNVScan.

This study analysed CNV on three genes known to have different level of amplification and which are located either in the nuclear, apicoplast or mitochondrial genomes. The results showed a correlation between PlasmoCNVscan and the qPCR experiments, usually used for identification of locus specific amplification/deletion. The use of such a tool for the exploration of adaptive phenomena based on whole genome data is then discussed.

Methods

DNA

Real time PCR and whole genome analysis were carried out on the same DNA extracted from samples of *P. falciparum* collected in Cambodia between 2010 and 2014 and adapted to culture. DNA extraction was performed using QIAamp DNA Blood Kit (Qiagen ©).

qPCR

The protocol for qPCR copy number evaluation used in this study was based on the WWARN (MOL-05) procedure: “Copy number estimation of *P. falciparum* *pfmndr1* v1.1”. Relative quantification was performed by using “PCR Applied Biosystem ViiA 7®” and the Taqman® technologies (Thermo Fisher©).

An evaluation of the *pfmndr1*, *clcp* (PFC10_API0060) and *cytochrome b* genes was performed because they are all known to have CNV and belong to the three genomes (respectively from nuclear, apicoplast and mitochondrial genomes). The reference gene selected was the nuclear beta-tubulin-encoding gene (PF10_0084). The primers and probe used for the qPCR are described in the Table 1.

All samples were analysed in triplicate. The confidence intervals on measures must be superior to 95 % for one triplicate and the Z-score, designating the deviation from a normal distribution, must be inferior to 1.75 (Life Technologies Corporation, 2011). All the samples results that did not meet these criteria were removed from the final results.

Whole genome

Whole-genome sequencing was performed on parasite DNA from Cambodian parasite isolates, using an Illumina paired-reads sequencing technology, as previously described [11].

PlasmoCNVScan

Read depth-based methods have recently become a major approach for estimating copy number [12]. The underlying concept of RD-based methods is that the depth of coverage in a genomic region is correlated with the copy number of the region; e.g., a lower than expected depth of coverage intensity indicates deletion and a higher than expected depth of coverage intensity indicates amplification [13]. The algorithm in classical RD-based methods relies heavily on the assumption that the sequencing process is uniform, i.e., the number of reads mapped to a region is assumed to follow a Poisson distribution and is proportional to the number of copies [12]. However, due to the GC content and “mapability”, this assumption is for the most part unrealistic. Moreover, the uneven representation of genomic regions in library preparation due to variability in DNA fragmentation may induce a bias [14].

In PlasmoCNVScan this assumption is by-passed using sequence pattern coverage across the overall exome. The reads must be correctly mapped onto a well-annotated reference genome. The main hypothesis is that the depth of coverage for each motif in the exome only depends on the sequence and thus has the same intensity. Here, a motif represents a subset of a fixed number

Table 1 The primers and probe used for copy number quantification

Name	Sequence	Gene amplification	Location
CytbF	5'GCACGCAACAGGTGCTTCTC 3'		Cytochrome b
CytbR	5'GACCCCATGGTAAGACATAACC 3'		Mitochondria
CytBP	5'(FAM)-CCATGATAATGGTAAACATATGAGTAATT-(TAMRA) 3'		
CLCPF	5'GGGCCTAGTGGACTGGTAA 3'	clcp	
CLCPR	5'CCAACATAACCAGGAGGTGAACC 3'		Apicoplast
CLCPP	5'(FAM)-CATATCAAATCTAATTAGTTCTTTCAGAAC-(TAMRA) 3'		
Mdr1F	5'TGCATCTAAAACGATCAGACAAA 3'	pfmdr1	Nuclear
Mdr1R	5'TCGTGTGTCATGTGACTGT 3'		
Mdr1P	5'(FAM)-TTAATAACCTGATCGAAATGAAACCTTG-(TAMRA) 3'		
TubF	5'AAAAATATGATGTGCGCAAGTGA 3'	Pftubulin	
TubR	5'AACTCCTTGTGGACATTCTCCT 3'		
TubP	5'(TET)-TAGCACATGCCGTTAAATCTTCCATGTCT-(TAMRA) 3'		

of nucleotides in the genome. The motif's coverage is the average coverage of this subset (see Fig. 1).

Firstly, the average frequency for each motif found across the whole exome was computed: this is the theoretical coverage for a motif. The observed coverage is the local coverage for a motif for each position (extracted from pileup file). Then, for each gene, using a sliding window, the ratio between observed coverage and theoretical coverage for each gene/position was computed. This ratio gives the estimated copy number variation for this region.

The algorithm was implemented in homemade software in C language called PlasmoCNVScan. PlasmoCNVScan use the external libraries gbf [15] under GNU GPL v2 licence and utash.under the revised BSD licence.

Optimising the size of the sequence length used for the motif

The length of the motif is arbitrary, but clearly a motif of size 1 nucleotide will completely cover the genome but will yield no information on intra-genomic variation, whereas a size of hundred nucleotides will lead to little coverage and huge variation in the coverage across the genome. The motif size was increased from 1 until the variance in the coverage among intra-genomic region increased. Using the reference genome, *P. falciparum* clone 3D7, the optimal number of nucleotides for the motif was assessed. As can clearly be seen in Fig. 2, the variance increases significantly after a motif length of 6 nucleotides. The optimal motif size appears to be 5 or 6 nucleotides. The size of the motif was set to 6.

PlasmoCNVScan versus benchmark softwares

The dataset was tested for *pfmdr1* gene with two programs for detecting copy number variation using next

generation sequencing data. CNV-seq [16], which is widely used software in case-control studies, and CNVnator [17], which uses a similar approach to calculate RD signal and correct the GC-bias.

Statistical analysis

The qPCR results were considered as reference and the Pearson test was used to calculate the measure of the linear correlation (dependence) between the two variables qPCR and PlasmoCNVScan or CNVnator software, giving a value between +1 and -1 inclusive, where 1 is total positive correlation.

Results

According to the results of the copy number obtained for 19 isolates (*cytochrome b* gene, mitochondrial genome), 21 isolates (*clcp* gene, apicoplast genome) and 42 isolates (*pfmdr1* gene, nuclear genome) with real-time PCR, a correlation line was established with the results from PlasmoCNVScan tool on illumina FASTq files. As can be seen in Fig. 3, R^2 values for the two types of extra nuclear genome and for nuclear genome are greater than 0.8. Moreover, the equation obtained type $y = ax + b$ has a factor "a" close to 1 with a very low b value, tending towards the type $y = x$; thus both methods are proportional to each other and tend to be similar.

PlasmoCNVScan vs CNV-seq and CNVnator

CNV-seq

As CNV-seq method is conceptually derived from array comparative genomic hybridization (aCGH), two sets of reads mapped onto the same reference genome from the same flow cell is needed. CNV-seq fails to detect CNV on all isolates, because 3D7, used as a reference, has been

PlasmoCNVScan Algorithm

PlasmoCNVScan is a C/C++ software to normalize read depth along the genome. The underlying concept of ReadDepth-based methods is that the depth of coverage in a genomic region is correlated with the copy number of the region.

Firstly, we compute the average frequency for each motif found across the whole exome: this is the theoretical coverage for a motif. We define the observed coverage as the local coverage for a motif for each position. Then, for each gene we use a sliding window and compute the ratio between observed coverage and theoretical coverage for each gene. This ratio gives us the estimated copy number variation for this small region.

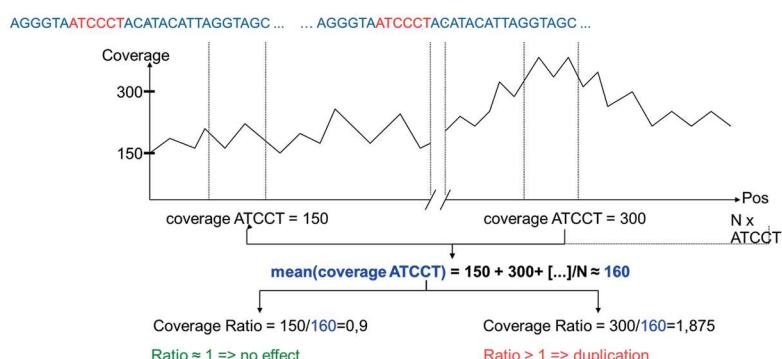


Fig. 1 PlasmoCNVScan algorithm

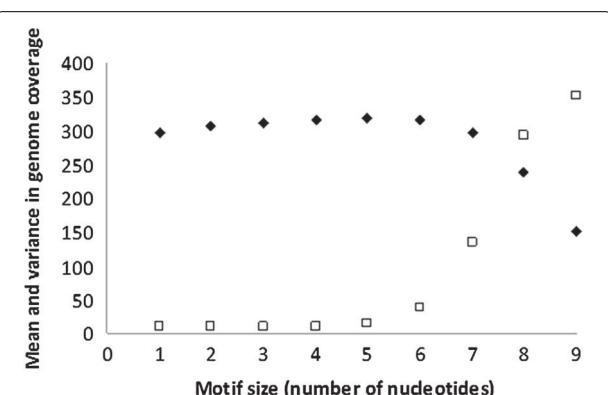


Fig. 2 Motif size, mean and variance relation. Mean coverage of the genome is represented as filled squares and variance coverage of the genome is represented as open squares. Variance is divided by 100 for clarity

sequenced on a different flow cell to the other isolates. To avoid this problem, there is a need to include a reference isolate in each of the flow cells used, which becomes prohibitively expensive.

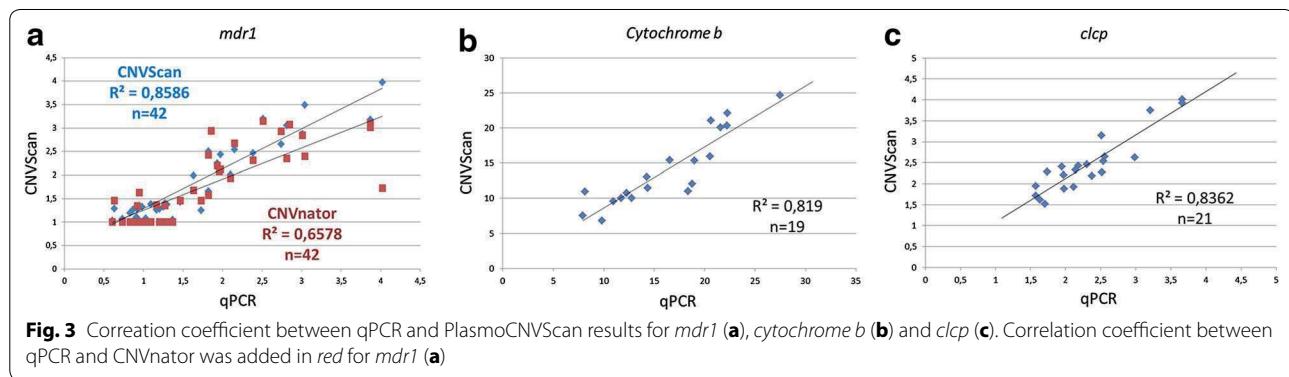
CNVnator

CNVnator is able to discover CNVs in a vast range of sizes, from a few hundred bases to megabases in length for a single genome. The correction of GC-bias is based under the observation that the RD signal and GC content are correlated. Strikingly, CNVnator had a lower correlation with qPCR than PlasmoCNVScan ($R^2 = 0.65$, $N = 42$ Fig. 3).

Discussion

The overall (A + T) composition is 80.6 % in the *P. falciparum* genome and increases to ~90 % in introns and intergenic regions [18], resulting in very high similarity among non-coding regions. This introduces an important bias for CNV identification using NGS data. In coding regions, the GC content is higher and the coverage is likely to be higher and more specific. This heterogeneity in the GC content between coding and non-coding sequences led us to compute the average coverage for exons only.

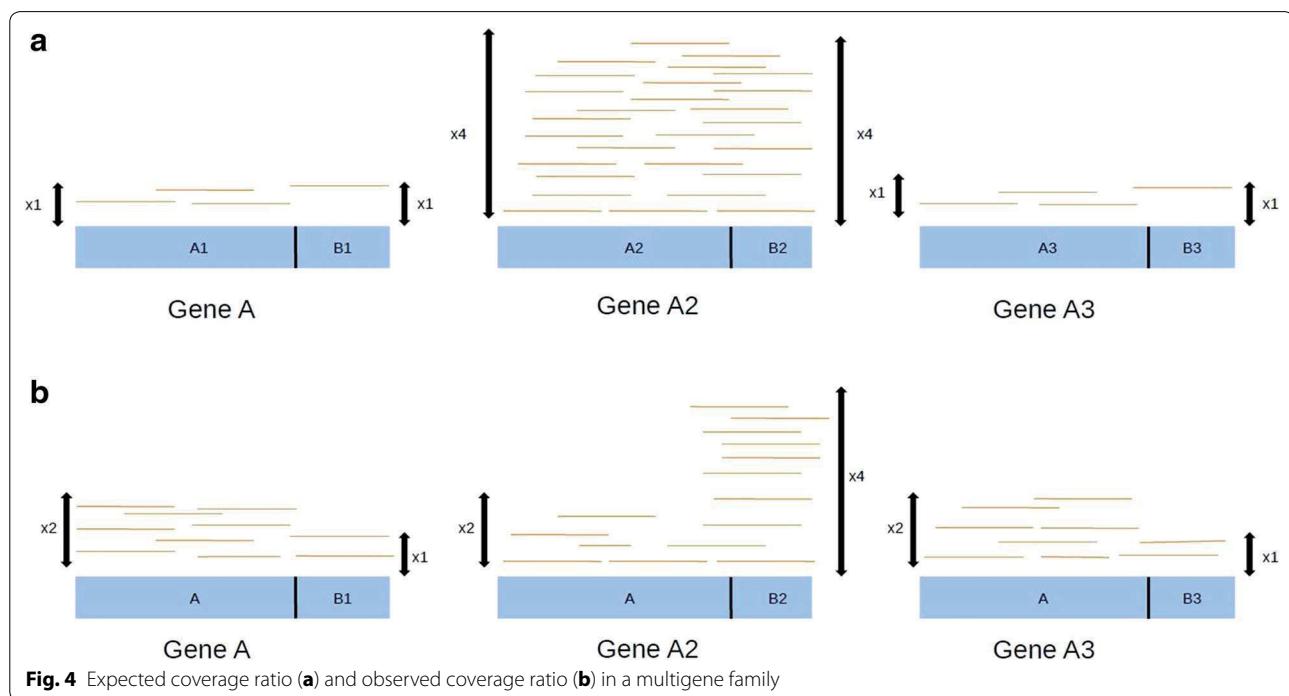
For computing the CNV on a single sample using PlasmoCNVScan, only a BAM file (which is converted in pileup file) is necessary, along with the reference genome



(fasta file) and a gff file. Given mapped reads, the efficient implementation of PlasmoCNVScan allowed a non IT specialist to perform whole-exome analysis of *P. falciparum* within a few minutes on a single 3.3-GHz Intel Core 3 Duo CPU. The RD signal is normalized with the genome itself. PlasmoCNVScan is thus able to compare different CNV exomes from different experiments. The results show very good correlation with the qPCR results, with R^2 value above 0.8 for all the three genes explored irrespective of the CNV range (from 1 to 30 in the case of *cytochrome b* mitochondrial gene).

The main limitation of the algorithm is that when facing multigene families biases could appear for gene amplification detection or for the precise identification of the gene really amplified. Figure 4 shows an example

in the case of a multigene family. The three genes share a common sequence (A) and a variable sequence (B1, B2, B3). The reality is shown in Fig. 4a: genes A1 and A3 are not amplified, gene A2 harbors four copies, thus the ratio given by PlasmoCNVScan should be four. The observed computed ratio is shown in Fig. 4b. Because of the A common sequence, reads are equally distributed among the multigene family and the computed ratio is $2 : (4+1+1)/3 = 2$. The computed ratio for specific regions (B1, B2, B3) are correct. In the case presented in this paper the *clcp*, *cytochrome b* and *pfdm1r1* genes showed no significant common nucleotide sequences with other genes to scramble information. Confirmation by qPCR targeting specific areas of the studied genes would circumvent this problem.



However when working with polyclonal infections, which is a very common situation in Africa, the same problem may arise in the case of mixed infections with different parasites that do not possess the same CNV profile. In this case the qPCR will be of no help.

Conclusions

The aim of this study was to test the ability of the algorithm to calculate the CNVs based on a whole genome sequencing with small reads (FASTQ). Thus the authors chose to work on clonal isolates directly isolated from the field (not reference strains). The Cambodian isolates were previously culture adapted (only for several cycles) before DNA extraction, likely leading to the removal of minor clones. The exome approach generates even more accurate data because of the higher GC content of the coding regions than in the intergenic regions, and, of course, expressed genes have much less similarity among them.

The strong correlation found between classical qPCR and PlasmoCNVScan opens the way for a systematic screening of CNVs changes on whole exomes. The global analysis of changes in the *P. falciparum* exome CNVs is beyond the scope of this article, but it is hoped that PlasmoCNVScan can be a useful tool to explore *P. falciparum* genomic adaptation in the face of ecological changes: drug pressure, decreased transmission, reduction of the parasite population size (transition to pre-elimination endemic area).

Authors' contributions

JB carried out the PlasmoCNVScan algorithm and software, AL, NK, VD and EL carried out the qPCR experiments. LG and RP carried out the statistical analysis of the algorithm. DM and FA supervised, carried out and coordinated the field collections and cultures of parasites. LM, BW and CB carried out the whole genome sequencing by Illumina method. All the authors read and approved the final manuscript.

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Acknowledgements

This study benefited from Whorld Health Organisation within the Karma project. This work was supported in part by BioMerieux.

Competing interests

The authors declare that they have no competing interests.

Received: 8 December 2015 Accepted: 31 March 2016

Published online: 12 April 2016

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Les combinaisons thérapeutiques à base d'artémisinine (ou CTAs) sont une des clés de voûte des stratégies actuelles de lutte contre le paludisme : ces thérapies ont en effet joué un rôle important dans la réduction de l'impact du paludisme au cours de la dernière décennie. Cependant, ces progrès sont aujourd'hui compromis par l'émergence de parasites *Plasmodium falciparum* résistants aux dérivés de l'artémisinine (ART). Les premiers parasites résistants ont été détectés pour la première fois en 2008 dans l'ouest du Cambodge, puis dans plusieurs pays avoisinants d'Asie du Sud-Est. Le problème de la multirésistance aux antipaludiques s'est récemment aggravé au Cambodge : plusieurs études ont rapporté l'émergence de parasites résistants à la pipéraquine (PPQ), la dernière génération de molécule partenaire utilisée en combinaison avec la dihydroartémisinine, entraînant des taux alarmants d'échecs cliniques. Pour préserver l'efficacité de ces combinaisons, la surveillance et la compréhension de ces deux types de résistance sont cruciales afin d'éviter la dissémination de parasites multirésistants en dehors d'Asie du Sud-Est, et particulièrement en Afrique où les conséquences sanitaires seraient désastreuses. À cette fin, le développement de nouveaux outils est nécessaire pour étudier les mécanismes biologiques et moléculaires impliqués dans la résistance aux CTAs et pour surveiller la distribution géographique des parasites multirésistants.

Dans la première partie de cette thèse, axée sur la résistance à l'artémisinine, nous présentons tout d'abord la découverte de mutations au sein du gène K13, marqueur moléculaire pour la résistance à l'ART. Puis nous confirmons leur rôle comme déterminant majeur de cette résistance. Enfin, nous analysons l'étendue de cette résistance au travers d'une cartographie mondiale des polymorphismes de K13. Dans la seconde partie de cette thèse, nous présentons nos travaux portant sur l'émergence de la résistance à la pipéraquine au Cambodge, en confirmant dans un premier temps que des parasites multirésistants à l'ART et à la PPQ circulent désormais dans le pays. Puis en détaillant la mise en point d'un nouveau test phénotypique pour la détection des parasites PPQ-résistants, le Piperaquine Survival Assay ou PSA. Pour finir, nous exposons la découverte de l'amplification du gène PfPM2 comme marqueur moléculaire pour la résistance à la PPQ.

Artemisinin-based combination therapies (ACTs) are one of the pillars of the current strategies implemented for fighting malaria. Over the last decade, ACTs have played a major role in decreasing malaria burden. However, this progress is being jeopardized by the emergence of artemisinin-resistant *Plasmodium falciparum* parasites. Artemisinin (ART) resistance was first detected in western Cambodia in 2008 and has since been observed in neighboring countries in Southeast Asia. The problem of antimalarial drug resistance has recently worsened in Cambodia, with reports of parasites resistant to piperaquine (PPQ), the latest generation of partner drug used in combination with dihydroartemisinin, leading to worrying rates of clinical treatment failure. The monitoring and the comprehension of both types of resistance are crucial to prevent the spread of multi-drug resistant parasites outside Southeast Asia, and particularly to Africa, where the public health consequences would be catastrophic. To this end, new tools are required for studies of the biological and molecular mechanisms underlying resistance to antimalarial drugs and for monitoring the geographic distribution of the resistant parasites.

In the first section of this thesis, centered on artemisinin resistance, we first present the discovery of mutations within the K13 gene as a molecular marker for ART resistance. Then we confirm their role as a major determinant of such a resistance. Finally, we analyze the extent of ART resistance through a global mapping of K13 polymorphisms. In the second section, we present our work on the emergence of piperaquine resistance in Cambodia, by initially confirming that multiresistant parasites now circulate in the country. Then we detail the development of a new phenotypical test for the detection of PPQ-resistant parasites, the Piperaquine Survival Assay or PSA. Lastly, we report the discovery of the PfPM2 gene amplification as a candidate molecular marker for PPQ-resistance.