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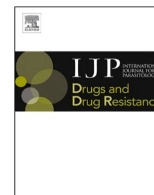

Longitudinal *ex vivo* and molecular trends of chloroquine and piperaquine activity against *Plasmodium falciparum* and *P. vivax* before and after introduction of artemisinin-based combination therapy in Papua, Indonesia

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A R T I C L E I N F O

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A B S T R A C T

Drug resistant *Plasmodium* parasites are a major threat to malaria control and elimination. After reports of high levels of multidrug resistant *P. falciparum* and *P. vivax* in Indonesia, in 2005, the national first-line treatment policy for uncomplicated malaria was changed in March 2006, to dihydroartemisinin-piperaquine against all species.

This study assessed the temporal trends in *ex vivo* drug susceptibility to chloroquine (CQ) and piperaquine (PIP) for both *P. falciparum* and *P. vivax* clinical isolates collected between 2004 and 2018, by using schizont maturation assays, and genotyped a subset of isolates for known and putative molecular markers of CQ and PIP resistance by using Sanger and next generation whole genome sequencing.

The median CQ IC_{50} values varied significantly between years in both *Plasmodium* species, but there was no significant trend over time. In contrast, there was a significant trend for increasing PIP IC_{50}s in both *Plasmodium* species from 2010 onwards. Whereas the South American CQ resistant 7G8 *pfcrt* SVMNT isoform has been fixed since 2005 in the study area, the *pfmdr*1 86Y allele frequencies decreased and became fixed at the wild-type allele in 2015. In *P. vivax* isolates, putative markers of CQ resistance (no *pvcrt-o* AAG (K10) insertion and *pvmdr*1 Y967F and F1076L) were fixed at the mutant alleles since 2005. None of the putative PIP resistance markers were detected in *P. falciparum*.

The *ex vivo* drug susceptibility and molecular analysis of CQ and PIP efficacy for *P. falciparum* and *P. vivax* after 12 years of intense drug pressure with DHP suggests that whilst the degree of CQ resistance appears to have been sustained, there has been a slight decline in PIP susceptibility, although this does not appear to have reached clinically significant levels. The observed decreasing trend in *ex vivo* PIP susceptibility highlights the importance of ongoing surveillance.

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1. Introduction

Malaria remains one of the most important tropical diseases globally, causing more than 200 million clinical cases per year and more than 400 deaths (WHO, 2019). The emergence and spread of resistant *Plasmodium* parasites are a major threat to disease control and elimination efforts. The Greater Mekong Subregion (GMS) has been the epicentre of the emergence of *P. falciparum* resistance with parasites now being resistant to almost all antimalarials used to date (Dondorp et al., 2009; Noedl et al., 2009; Wongsrichanalai et al., 2002), including the artemisinin-based combination therapy (ACT) regimen dihydroartemisinin-piperaquine (DHP) (Saunders et al., 2014). Standardized clinical and *in vitro* protocols have been developed for *P. falciparum*, which have enhanced the surveillance of resistant parasites and facilitated discovery and validation of the underlying genetic mechanisms of resistance and complementary molecular surveillance. In contrast, the surveillance of drug resistant *P. vivax* is constrained to clinical efficacy studies that are confounded by various host factors such as host immunity and drug metabolism, as well as difficulties in pinning down the origin of recurrent parasites as recrudescent (drug resistant), relapsing (originating from hypnozoites), or new infections (Popovici and Ménard, 2015).

P. falciparum can be maintained in continuous *in vitro* culture, making it amenable to drug susceptibility testing by exposure to the presence of serial drug concentrations. In contrast, *P. vivax* preferentially invades young red blood cells and its limited reproductive capacity has so far prevented continuous *in vitro* culture. Although drug susceptibility of *P. vivax* has been assessed using short-term *ex vivo* culture, such studies generally require blood collected directly from patients with malaria and thus, is limited to field sites with good laboratory infrastructure. The life cycle stage composition of *P. vivax* infections is highly variable and can potentially bias *ex vivo* drug susceptibility testing results (Russell et al., 2008; Thomson-Luque et al., 2019). The inability to propagate *P. vivax* parasites in continuous *in vitro* culture has also severely hampered investigation of the molecular determinants of drug resistance of the parasite. To date, putative determinants of drug action and resistance have been identified by assessing *ex vivo* drug susceptibility profiles (Russell et al., 2008), adaptation and propagation of *P. vivax* isolates in non-human primate hosts (McCallum et al., 2020), expression of orthologous genes identified in *P. falciparum* in heterologous systems (Sa et al., 2006), and correlation of putative molecular markers and *in vivo* or *ex vivo* CQ-resistant phenotypes (Marfurt et al., 2008; Suwanarusk et al., 2007). However, results of validation studies have been inconclusive and hence, a consensus set of resistance markers does not exist.

In Papua Indonesia, high levels of antimalarial drug resistance have emerged in both *P. falciparum* and *P. vivax*. Clinical trials conducted in 2004 demonstrated that more than 48% of patients with *P. falciparum* malaria treated with sulfadoxine-pyrimethamine plus chloroquine and 65% of patients with *P. vivax* malaria treated with chloroquine alone had recurrent infections within 28 days (Ratcliff et al., 2007). In March 2006, antimalarial treatment guidelines were revised to a universal policy of dihydroartemisinin-piperaquine (DHP) for uncomplicated malaria due to any species.

This study aimed to assess the temporal trends in *ex vivo* drug susceptibility to chloroquine (CQ) and piperaquine (PIP) for both *P. falciparum* and *P. vivax* clinical isolates collected between 2004 and 2018 and associated patterns of known and putative molecular markers of antimalarial drug resistance.

2. Materials and methods

2.1. Study area and field sample collection

The study was conducted in Timika, southern Papua Province in Eastern Indonesia, between March 2004 and October 2018. Patients presenting to the Rumah Sakit Mitra Masyarakat (RSMM) and Rumah Sakit Umum Daerah (RSUD) hospitals were screened for eligibility criteria and were asked to enrol into the study if they had a microscopically confirmed *P. falciparum* or *P. vivax* mono-species infection and a parasitaemia between 2000 and 80,000 parasites/μL. Between March 2004 to December 2008, *Plasmodium* spp. isolates were processed from all eligible patients, but from December 2008 isolates were processed only if the blood film at screening had at least 66% of asexual forms at ring stage. Patients were excluded from the study if they were treated with CQ in the last 30 days or any other antimalarials in the previous two weeks or had haemoglobin levels below 5 g/dL. After written informed consent was obtained, 5 mL of venous blood was collected and processed immediately. Host white blood cell depletion was performed by using either CF11 cellulose filtration (Richards and Williams, 1973); March 2004–April 2015) or commercially available Plasmodipur® filters (Europroxima B.V., Arnhem, The Netherlands; May 2015–October 2018) according to the manufacturer's instructions. Packed infected red blood cells (iRBCs) were used for all *ex vivo* drug susceptibility assays.

2.2. Compounds and drug plate preparation

A range of novel antimalarial agents were assessed and have been reported previously (Baragaña et al., 2015; Brunschwig et al., 2018; Kuhen et al., 2014; Le Bihan et al., 2016; Marfurt et al., 2011a; Marfurt et al., 2011b; Marfurt et al., 2012b; Nilsen et al., 2013; O'Neill et al., 2017; Phillips et al., 2016; Price et al., 2010; Vaidya et al., 2014; Wirjanata et al., 2015a; Wirjanata et al., 2015b; Xue et al., 2019). All plates also contained the two standard antimalarials chloroquine (CQ) and piperaquine (PIP). CQ and PIP were sourced from the WWARN QA/QC Reference Material Programme (Lourens et al., 2010). Drugs were prepared as 1 mg/mL solution in dimethyl sulfoxide (DMSO) and drug plates pre-dosed by diluting the compounds in 50% methanol followed by lyophilisation and storage at 4 °C. Each 96- well drug plate contained 11 serial 2-fold dilutions in duplicate with a maximum concentration of 2993 nM for CQ and 1146 nM for PIP.

2.3. Ex vivo drug susceptibility assay

CQ and PIP susceptibilities of *Plasmodium* laboratory strains and clinical isolates were measured using a modified schizont maturation assay as described previously (Marfurt et al., 2012a; Wirjanata et al., 2017). Drug susceptibility profiles were presented as inhibition of parasite growth from ring to schizont stage. Two hundred microliters of a 2% hematocrit blood medium mixture (BMM), consisting of RPMI 1640 medium supplemented with 10% human serum (*P. falciparum*) or McCoy's 5A medium (Life Technologies, VIC, Australia) supplemented with 20% human serum (*P. vivax*), were added to each well of the pre-dosed 96-well drug plates. The parasites were cultured in a candle jar at 37.0 °C for 35–50 h and growth in the control well monitored. Incubation was deemed successful and stopped when ≥40% of ring-stage parasites had reached the mature schizont stage (≥4 distinct nuclei per parasite) in the drug-free control well. The assays were harvested by preparing thick blood films from each well of the plates; slides were stained with 5% Giemsa solution for 30 min and examined microscopically. The number of schizonts per 200 asexual stage parasites was determined for each drug concentration and normalized to the control well (Marfurt et al., 2011a, 2011b).

2.4. Quality control (QC) procedures

Drug plate quality was assessed using duplicate independent schizont maturation assays with the chloroquine-resistant *P. falciparum* laboratory strain K1 and the chloroquine-sensitive strain FC27. The *P. falciparum* strains (BEI Resources, ATCC Manassas, Virginia, US) were maintained using the method of Trager and Jensen (Trager and Jensen,

1976) in complete malaria culture medium (CMCM) consisting of RPMI 1640 (Life Technologies, Victoria, Australia) supplemented with 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES; Life Technologies, Victoria, Australia), 2 mM L-glutamine (Life Technologies, Victoria, Australia), 40 mg/L gentamycin (Pfizer, NSW, Australia), 11 mM glucose (Sigma-Aldrich, NSW, Australia), and 10% O^+ human serum. Cultures were incubated at 2% hematocrit in a candle jar at 37 °C. Synchronization of parasite cultures was conducted by D-sorbitol (Sigma-Aldrich, NSW, Australia)-treatment as previously described (Lambros and Vanderberg, 1979).

Microscopy slide reading was quality assured by randomly selecting 2 drugs per *Plasmodium* isolate, which were re-read by a second microscopist. If the raw data derived by the two microscopists resulted in a two-fold shift in the IC_{50} estimates of the selected drugs, the assay including all standard drugs and experimental compounds was re-read by a second reader and the results compared. Discrepant results were resolved by a third reading by an expert microscopist.

Quality assurance testing between 2004 and 2018 confirmed expected differential CQ susceptibility for K1 (CQ IC50 range: 78.0–271.2 nM for the CQ-resistant P. falciparum laboratory strain) and FC27 (CQ IC50 range: 5.1–57.5 nM for the CQ-sensitive strain FC27). Susceptibility to PIP was similar in both strain and constant over the years (PIP IC_{50} range: 10.4–90.3 nM for both strains). To control for potential variability in drug plate preparation and starting conditions, a sensitivity analysis of temporal trends was performed after excluding drug plates for which the IC_{50} values for control strains were >1.5 fold higher than the overall median of all IC_{50} values, and only including isolates that had >90% ring stage parasites at the start of the assay and an assay duration of 34–42 h.

2.5. Genotyping of molecular markers of resistance

For *P. falciparum*, analysis of markers of CQ resistance focused on single nucleotide polymorphisms (SNPs) in the *P. falciparum* chloroquine resistance transporter gene (*pfcrt*; amino acid changes at C72S, V73I, M74I, N75D/K, K76T, H97L/Y, T152A, 163R, A220S, Q271E, N326D/S, T333A/S, I356L/T, and R371) and multidrug resistance 1 gene (*pfmdr*1; amino acid changes at N86Y, Y184F, S1034C, N1042D, and D1246Y). Between 2004 and 2006, *pfcrt* was genotyped using a protocol described by Fidock and colleagues (Fidock et al., 2000) and *pfmdr*1 was genotyped using the PCR-RFLP method described by Duraisingh et al. (2000). *Pfcrt* and *pfmdr*1 genotyping data were available for 17 and 89 isolates, respectively, between 2005 and 2006. Forty-six and 89 *P. vivax* isolates were genotyped for the *pvcrt-o* AAG (K10) insertion in exon 1 and *pvmdr*1 Y967F and F1076L, respectively (Suwanarusk et al., 2007). From 2011 onwards, 114 *P. falciparum* and 240 *P. vivax* isolates were genotyped from data derived by whole genome sequencing (WGS), as described previously (Auburn et al., 2018, 2019; Pearson et al., 2016, 2020). Putative markers of PIP resistance were derived for *pfcrt* SNPs at T93S, H97Y, C101F, F145I, I218F, M343L, C350R, G353V, and *exo*-E415G. *P. falciparum plasmepsin* 2 (*pfpm*2) copy number variants (CNVs) were determined by qPCR as described previously (Amato et al., 2017; Witkowski et al., 2017).

2.6. Data analysis

Raw drug response data were analysed using nonlinear regression analysis and the half maximal inhibitory concentration (IC_{50}) value derived applying an inhibitory sigmoid E_{max} model using WinNonlin (version 4.1; Pharsight Corporation, Mountain View, CA, US); March 2004–August 2015) or the In Vitro Analysis and Reporting Tool (IVART; August 2015–October 2018), a free and automated online platform to produce IC_{50} estimates (Woodrow et al., 2013). *Ex vivo* IC_{50} data were only used from predicted curves where the maximum percent growth (E_{max}) and minimum percent growth (E_0) were within 15% of 100 and 1, respectively.

IC_{90} values were estimated from IC_{50} values and the Hill slope (i.e., gamma) of the sigmoid drug response curve using the formula: $IC_{90} = (9^{\sqrt{\gamma}}) * IC_{50}$. Only data derived from drug plates passing independent quality control were included in the analysis. Previous threshold analysis has demonstrated that reliable drug response data from schizont maturation assays require >66% of parasites to be at ring stage at the start of the assay with a duration of drug exposure exceeding 34 h (Kerlin et al., 2012). IC_{50} values for CQ were also categorised to those above and below 100 nM for *P. falciparum* (Cerutti et al., 1999) and 220 nM for *P. vivax*, with the latter cut-off value determined at the 35th percentile of the continuous non-linear curve of ranked CQ IC_{50}s in 226 isolates from the region in 2005 when clinical CQ failure rates had reached 65% (Suwanarusk et al., 2007).

Statistical analysis was performed using STATA (version 15.1; Stata Corp., College Station, TX, US) and GraphPad Prism (version 8; GraphPad Software, Inc., San Diego, CA, US). The Mann-Whitney *U* test, Wilcoxon Signed Rank Test, Spearman Rank correlation, and Mann-Kendall test were used for nonparametric comparisons and correlations.

2.7. Ethical approval

Ethical approval for the study was obtained from the Eijkman Institute Research Ethics Commission, Eijkman Institute for Molecular Biology, Jakarta, Indonesia (EIREC-47, EIREC-67, and EIREC-75), the Ethics committee of the National Institute of Health Research and Development, Indonesian Ministry of Health, Jakarta, Indonesia (NIHRD: KS.01.01.6.591, NIHRD: KS.02.01.2.3.4579, NIHRD: KS.02.01.2.1.4042, NIHRD: KS.02.01.2.1.1615 and NIHRD:23 LB.03.02/KE/4099/2007), and the Human Research Ethics Committee of the Northern Territory (NT) Department of Health & Families and Menzies School of Health Research, Darwin, Australia (MSHR: 02/55, MSHR: 07/06, MSHR: 03/64, MSHR: 05/16, MSHR: 07/14 and HREC, 2010-1396).

3. Results

3.1. Ex vivo susceptibility to CQ and PIP

Between 2004 and 2018, 1478 patients were enrolled into the study and drug susceptibility testing attempted for 796 *P. falciparum* and 682 *P. vivax* isolates. A total of 1174 (79.4%) assays were successfully harvested with a success rate of 81.9% (652) for *P. falciparum* and 76.5% (522) for *P. vivax* isolates (Fig. 1).

The geometric mean parasitaemia in the successfully harvested isolates ranged from 11,662 to 55,405 asexual parasites μl^{-1} in *P. falciparum* isolates and 8516 to 33,514 parasites μl^{-1} in *P. vivax* isolates, with no significant variation over the study period. At the start of the assay, *P. falciparum* isolates were composed of 100% ring stages in almost all cases, whereas for *P. vivax* the median percentage of ring stages varied from 45% in 2004 to ≥90% from 2007 (Supplementary Fig. 1). The median duration of the assay for both species ranged from 20 to 30 h in the first three years 2004–2006 but exceeded 40 h from 2007 onwards (Supplementary Fig. 2).

Overall, acceptable P. falciparum IC_{50} estimates were available in 97.9% (462/472) isolates for CQ and 97.7% (461/472) for PIP. The corresponding values for *P. vivax* were 97.7% (344/352) for CQ and 96.9% (341/352) for PIP, respectively (Fig. 1). The IC_{50} values for CQ and PIP are presented in Table 1 and Figs. 2 and 3; the corresponding IC_{90} values in Table 2 and Supplementary Figs. 3 and 4.

For the 344 *P. vivax* isolates with acceptable CQ data, the median IC_{50} was 55.4 nM (range: 4.6 to 1411.6; Table 1, Supplementary Fig. 5) and the median IC_{90} was 125.5 nM (range: 7.6 to 12,127.9; Table 2, Supplementary Fig. 6). A total of 28.8% (99/344) of isolates had an IC_{50} greater than 100 nM and 5.8% (20/344) an IC_{50} greater than 220 nM (Suwanarusk et al., 2007). The corresponding values for the 462 *P. falciparum* isolates were a median IC_{50} of 70.2 nM (range: 5.2 to

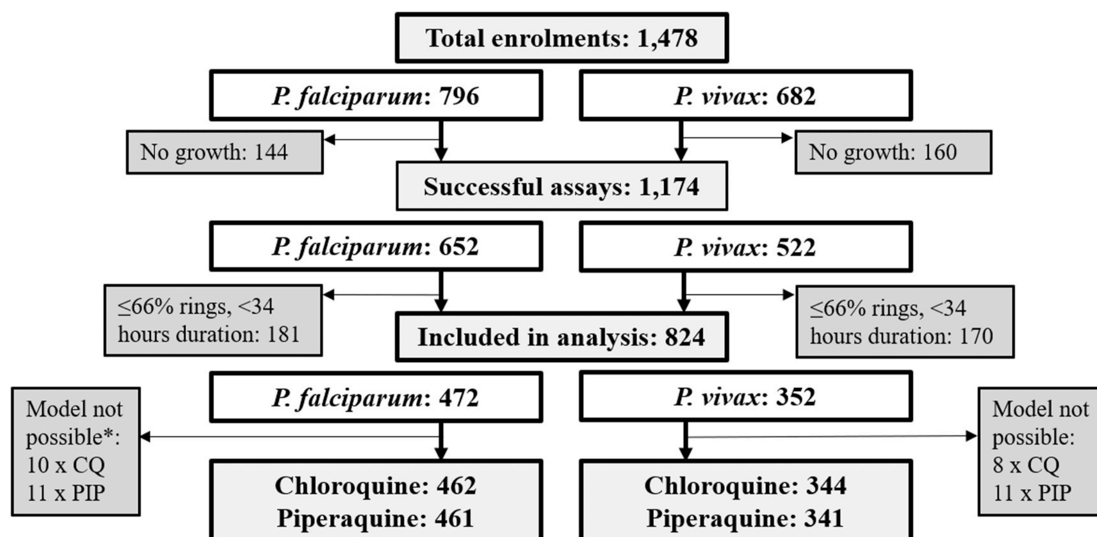

Fig. 1. Study profile for *ex vivo* drug susceptibility testing for chloroquine (CQ) and piperaquine (PIP) in clinical *P. falciparum* and *P. vivax* isolates. Footnote: * The drug response data could not be fitted to a sigmoid E_{max} model.

Table 1
Ex vivo dug susceptibility (IC_{50s}) for *P. falciparum* and *P. vivax* isolates collected and tested between 2004 and 2018.

		Chloroquine IC_{50} (nM) Median (range)				Piperaquine IC_{50} (nM) Median (range)		
Year	n	*P. falciparum*	n	*P. vivax*	n	*P. falciparum*	n	*P. vivax*
2004	8	46.8 (42.6–215.8)	2	16.3 (14.9–17.7)	8	13.2 (6.3–15.0)	0	
2005	16	50.4 (5.2–97.6)	7	25.5 (12.3–345.7)	14	6.9 (4.8–18.7)	4	8.8 (4.7–12.2)
2006	27	42.3 (6.7–104.3)	12	56.3 (4.6–1411.6)	24	15.7 (5.0–36.5)	13	19.5 (1.8–46.4)
2007	35	42.7 (11.9–127.6)	27	43.2 (10.0–855.2)	34	12.3 (2.7–46.5)	26	15.8 (5.4–49.1)
2008	41	36.3 (6.4–218.5)	35	51.0 (8.8–275.4)	41	11.8 (.3–63.9)	35	18.2 (3.4–66.9)
2009	39	91.4 (37.3–336.1)	45	68.6 (12.2–386.5)	39	19.8 (4.0–58.3)	45	17.0 (1.3–93.4)
2010	24	100.2 (5.8–265.8)	13	25.7 (13.1–80.8)	24	14.3 (6.4–79.0)	13	13.3 (5.5–25.9)
2011	44	65.7 (10.9–200.4)	45	43.3 (9.0–334.5)	44	27.5 (10.0–121.2)	45	17.2 (3.5–102.8)
2012	55	94.7 (28.4–279.0)	36	105.2 (26.2–383.4)	55	25.1 (5.2–58.1)	36	15.5 (3.4–75.3)
2013	21	80.1 (32.0–163.6)	37	126.3 (13.4–254.6)	21	22.6 (7.8–56.8)	37	27.4 (3.5–121.3)
2014	43	102.9 (20.3–247.2)	13	95.3 (38.6–783.8)	48	41.6 (4.2–86.5)	18	45.3 (4.2–109.6)
2015	32	95.7 (13.9–444.9)	22	54.1 (11.8–385.8)	32	32.5 (6.4–141.4)	22	41.7 (8.1–220.9)
2016	34	88.3 (37.2–283.2)	13	52.5 (7.0–125.3)	34	48.2 (8.3–130.0)	13	45.9 (15.0–134.8)
2017	34	83.7 (10.9–173.7)	30	46.9 (14.9–132.5)	34	22.6 (5.6–57.7)	27	20.3 (6.4–123.7)
2018	9	37.4 (11.5–116.1)	7	42.2 (24.0–77.8)	9	23.0 (8.5–42.9)	7	22.6 (13.5–60.3)
Tota	**462**	**70.2 (5.2–444.9)**	**344**	**55.4 (4.6–1411.6)**	**461**	**23.1 (0.3–141.4)**	**341**	**20.4 (1.3–220.9)**

Footnote: Data derived from *P. falciparum* and *P. vivax* isolates fulfilling acceptability criteria (>66% rings at the start of the assay and assay duration ≥34 h).

444.9) and IC_{90} of 139.7 nM (range: 7.3 to 747.2), with 31.0% (143/462) having a CQ IC_{50} greater than 100 nM. The median CQ IC_{50} values varied significantly between years ranging from 36.3 nM in 2008 to 102.9 nM in 2014 for *P. falciparum*, and from 25.5 nM in 2005 to 126.3 nM in 2013 for *P. vivax*, but there was no significant trend over time (Fig. 2).

Of the 341 *P. vivax* isolates with acceptable PIP data the median IC_{50} was 20.4 nM (range: 1.3 to 220.9) and the median IC_{90} was 38.6 nM (range: 3.0 to 449.9). The corresponding values for the 461 *P. falciparum* isolates were 23.1 nM (range: 0.3 to 141.4) and 44.9 nM (range: 1.3 to 456.6). There was a significant trend for increasing PIP IC_{50}s in both *P. falciparum* and *P. vivax* isolates from 2010 onwards (Mann-Kendall (MK) tau: 0.562, $p = 0.004$; and MK tau: 0.524, $p = 0.008$, respectively). There was a similar trend for PIP IC_{90}s (MK tau: 0.524, $p = 0.008$; and MK tau: 0.600, $p = 0.002$, respectively).

In the sensitivity analysis, excluding data from drug plates with outliers in quality control testing and only including isolates with ≥90% ring stage parasites at the start of the assay and an assay duration of ≥34 h, the temporal trends for PIP and CQ were similar. There was an increase in PIP IC_{50}s for *P. falciparum* (MK tau 0.543, $p = 0.006$) and *P. vivax* (MK tau 0.517, $p = 0.012$), but not for CQ IC_{50}s (MK tau 0.352, $p = 0.075$ and MK tau 0.257, $p = 0.198$, respectively; Supplementary Table 1).

3.2. Molecular markers of resistance

Pfcrt genotypes were available for 27% (131/478) of *P. falciparum* isolates. In the two years prior to the introduction of DHP (2004 and 2005), the CQ resistant genotype *pfcrt* 72**S**–V73-M74-N75-76**T**-220**S**-326**D**-333**A**/**S**–**S**-356**L**/**T** was present in all isolates and this remained stable over the study time period, except for 2 (1.5%) wild-type isolates, one enrolled in 2011 and the other in 2016 (Table 3). Genotyping data for *pfmdr*1 were available in 44% (203/478) of isolates. The mutant allele 86**Y** was present in 34% (12/35) of isolates in 2004 and 5% (2/37) in 2014 and thereafter, all parasites were wild-type N86. The allele frequencies of the two other mutant alleles 184**F** and 1042**D** increased from 60% (21/35) and 66% (23/35), respectively, prior to the introduction of DHP, to 94% (15/16) in 2017 (Table 4). None of the *P. falciparum* isolates genotyped for the putative PIP resistance markers had *pfpm*2 copy number amplification or any of the *pfcrt* alleles: 93**S**, 97**Y**, 101**F**, 145**I**, 218**F**, 343**L**, 350**R**, and 353**V**, or *exo*-415**G**.

Since the *pfcrt* 72**S**–V73-M74-N75-76**T**-220**S**-326**D**-333**A**/**S**–**S**-356**L**/

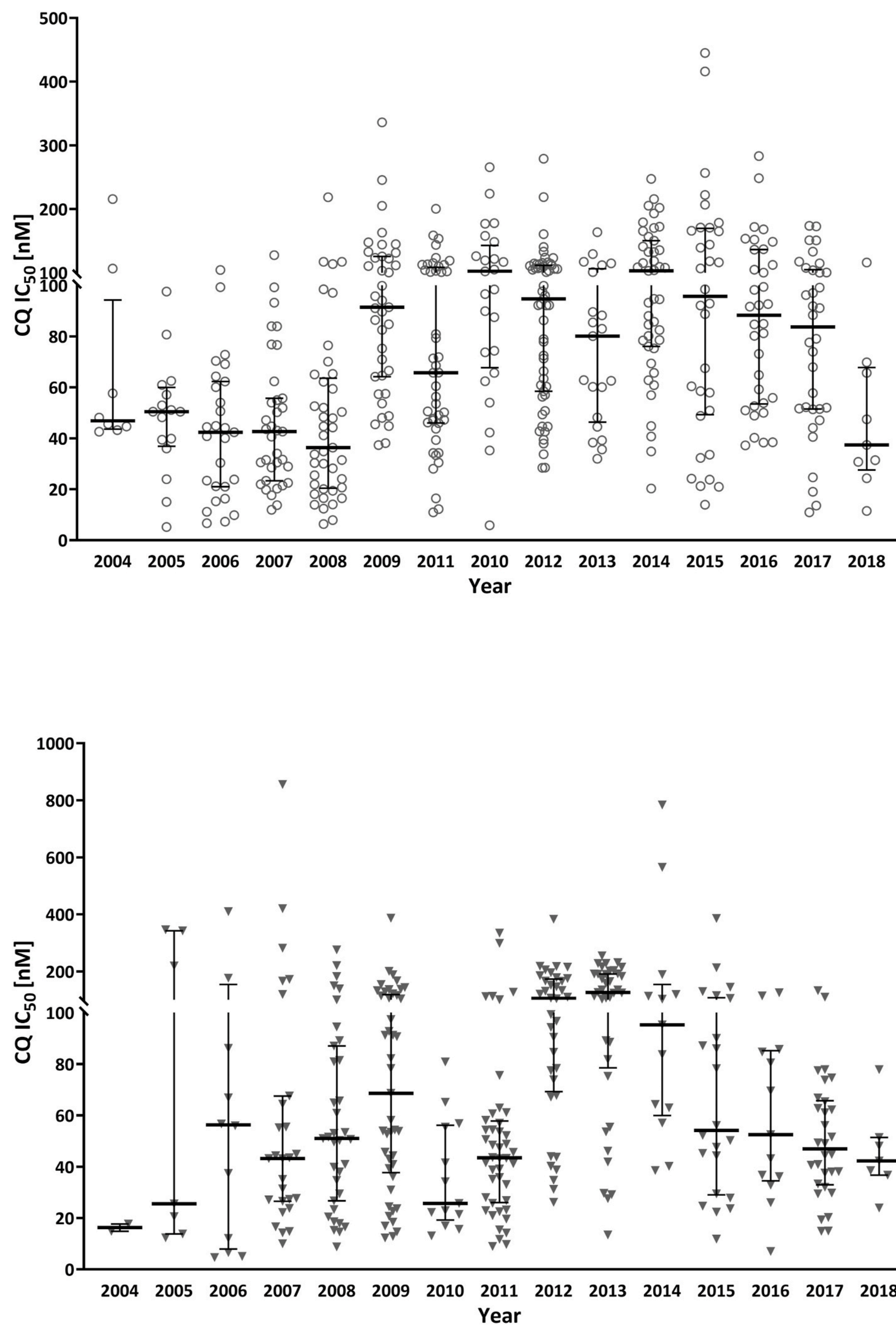

Fig. 2. Chloroquine susceptibility (IC_{50s}) for *P. falciparum* (open circles, top panel) and *P. vivax* (triangles, bottom panel).
Footnote: Data derived from *P. falciparum* and *P. vivax* isolates fulfilling acceptability criteria (>66% rings at the start of the assay and assay duration ≥34 h). The horizontal lines represent median value with vertical lines stretching between the 25th and 75th percentiles.

T was the prevailing genotype and putative PIP resistance markers were not present over the entire study period, genotype-phenotype association analyses were not possible.

Genotyping *pfmdr*1 was available in 39% (187/478) of *P. falciparum* isolates and seven genotypes were observed: N**FSD**D, NYS**DS**, NYSND, **YFSD**D, **YF**SND, **YY**S**D**D, and **Y**YSND. The median CQ IC_{50}s did not differ between any of these seven genotypes.

A total of 82% (286/350) and 378 *P. vivax* isolates (including 28 isolates without a valid *ex vivo* CQ susceptibility phenotype) were genotyped for the *pvcrt-o* AAG (**K10**) insertion and the *pvmdr*1 967 and 1076 alleles, respectively. The *pvcrt-o* K10 insertion in exon 1 was present in 2% (1/46) of samples in 2005–2006, but in none of the 240 samples after 2011. Conversely, the mutant *pvmdr*1 alleles 967F and 1076L were present in 96% (123/128) isolates collected between 2005 and 2006, and in all 240 isolates tested after 2011. Since these genotypes were fixed in the *P. vivax* population in the area, association analyses were not possible.

4. Discussion

This study presents a comprehensive longitudinal analysis of *ex vivo* CQ and PIP susceptibility in clinical *P. falciparum* and *P. vivax* field isolates in Papua, Indonesia, where multidrug resistant *P. falciparum* and *P. vivax* co-exist. The analysis comprises more than 1000 clinical isolates

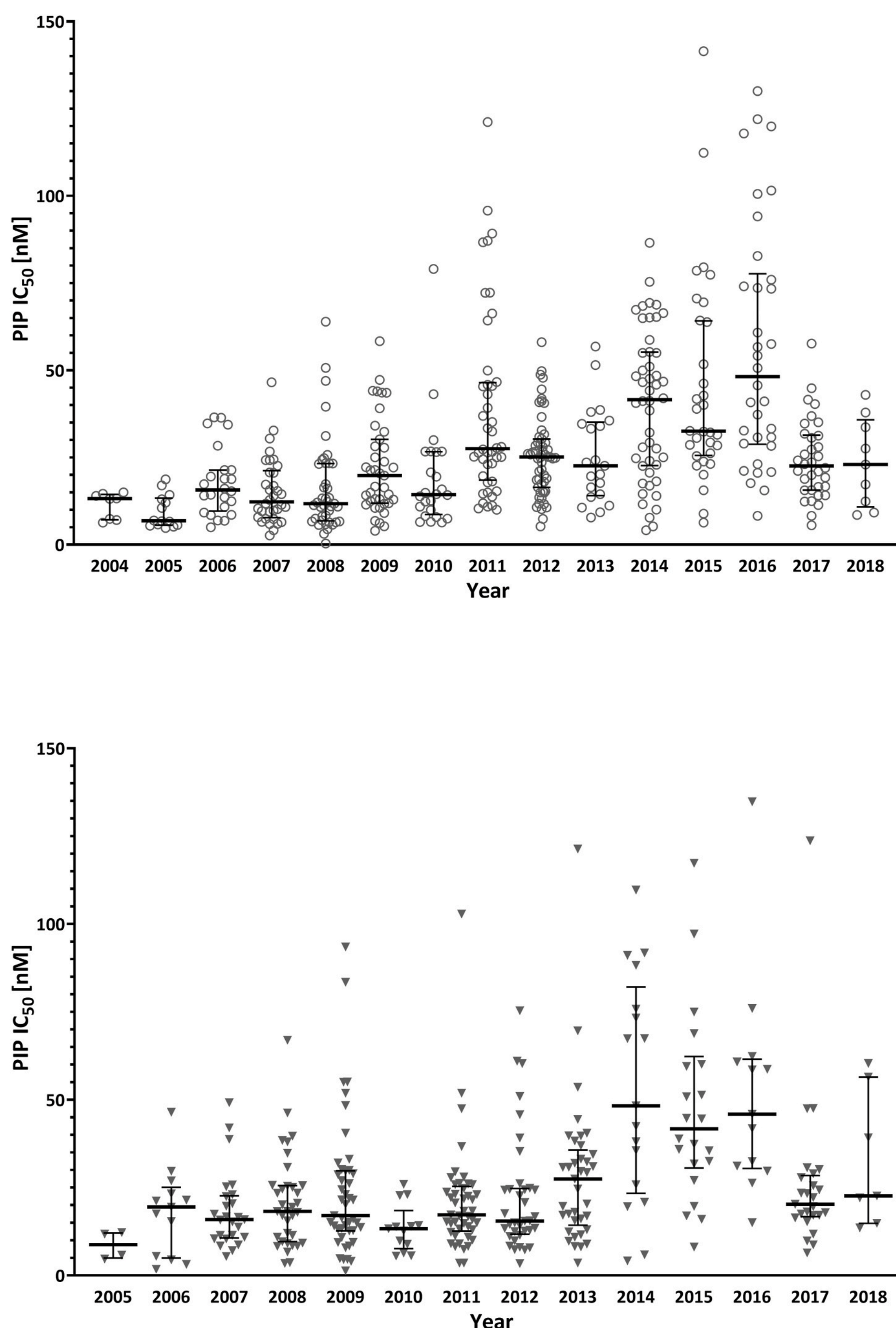

Fig. 3. Piperaquine susceptibility (IC_{50s}) for *P. falciparum* (open circles, top panel) and *P. vivax* (triangles, bottom panel).
Footnote: Data derived from *P. falciparum* and *P. vivax* isolates fulfilling acceptability criteria (>66% rings at the start of the assay and assay duration ≥34 h). The horizontal lines represent median value with vertical lines stretching between the 25th and 75th percentiles.

before and after a change in antimalarial treatment policy in 2006 from CQ for *P. vivax* and CQ + SP for *P. falciparum* to DHP for all patients with uncomplicated malaria due to any species. The results highlight different longitudinal trends. Although there was no change in CQ susceptibility, there was a trend for decreasing PIP susceptibility after 12 years of intense drug pressure with DHP.

The lack of a trend for increasing *ex vivo* CQ susceptibility after antimalarial policy change in 2006, contrasts observations in other areas where CQ susceptibility has been monitored before and after changes in national antimalarial policy (Frosch et al., 2014; Hayward et al., 2005). The end of CQ use was not implemented as radically in Papua, Indonesia, as it was in Malawi, Vietnam, and China where the drug was removed from both the public and private sectors. In Papua, more than 40% of patients with malaria continue to seek healthcare in the private sector where CQ remains readily available (Kenangalem et al., 2019). Hence, CQ pressure has likely continued throughout the study period and this may have maintained the continuation of reduced *ex vivo* CQ susceptibility.

The increasing PIP IC_{50}s and IC_{90}s in Papua for both *Plasmodium* species raises important questions. *Ex vivo* and *in vitro* data from the GMS, where DHP clinical failures have been reported, have shown a two-fold increase in *P. falciparum* PIP IC_{50}s (Agrawal et al., 2017; Chaorattanakawee et al., 2015; Dhingra et al., 2017; Hao et al., 2013; Mwai et al., 2009). Phenotypic characterization of Cambodian parasites

Table 2
Ex vivo dug susceptibility (IC_{90s}) for *P. falciparum* and *P. vivax* isolates collected and tested between 2004 and 2018.

		Chloroquine IC_{90} (nM) Median (range)				Piperaquine IC_{90} (nM) Median (range)		
Year	N	*P. falciparum*	n	*P. vivax*	n	*P. falciparum*	n	*P. vivax*
2004	8	139.7 (77.9–491.3)	2	63.2 (57.6–68.9)	8	29.1 (11.1–57.8)	0	
2005	16	98.8 (34.0–209.9)	7	524.3 (32.8–4491.4)	14	12.8 (9.7–34.3)	4	17.3 (11.1–25.4)
2006	27	70.9 (11.1–147.2)	12	167.9 (7.6–12127.9)	24	23.8 (8.7–71.9)	13	32.6 (7.1–76.6)
2007	35	81.4 (22.3–189.1)	27	114.8 (19.5–5457.7)	34	22.0 (5.3–58.3)	26	32.6 (10.8–116.9)
2008	41	71.6 (7.9–391.8)	35	117.1 (18.6–1114.0)	41	22.6 (1.3–346.0)	35	34.1 (5.4–115.1)
2009	39	167.5 (60.9–487.6)	45	151.6 (23.3–3118.5)	39	41.8 (6.2–242.4)	45	31.6 (3.0–151.9)
2010	24	168.5 (7.3–418.8)	13	47.4 (30.5–228.4)	24	21.5 (8.0–98.7)	13	17.2 (7.0–42.9)
2011	44	119.3 (13.6–250.2)	45	85.7 (16.9–2831.5)	44	57.1 (20.2–456.6)	45	32.7 (4.7–158.3)
2012	55	177.5 (47.5–648.0)	36	196.2 (42.5–986.4)	55	47.5 (16.3–206.3)	36	34.0 (11.2–134.2)
2013	21	179.7 (55.7–419.5)	37	272.3 (28.2–527.7)	21	58.1 (15.6–110.4)	37	39.8 (10.3–158.2)
2014	43	198.4 (29.8–449.3)	13	232.2 (50.1–4771.8)	48	82.7 (8.9–181.0)	18	105.9 (10.6–274.3)
2015	32	160.4 (17.3–554.3)	22	105.8 (29.5–615.4)	32	70.3 (28.5–311.2)	22	88.6 (20.3–449.9)
2016	34	175.0 (51.8–747.2)	13	106.8 (21.8–270.8)	34	115.8 (20.2–382.2)	13	99.4 (38.8–261.7)
2017	34	178.3 (19.5–550.4)	30	109.2 (26.1–433.0)	34	55.7 (16.0–130.5)	27	49.3 (21.3–200.9)
2018	9	91.6 (14.3–188.7)	7	95.9 (29.8–153.6)	9	43.3 (20.9–67.2)	7	46.9 (34.3–133.0)
Total	**462**	**139.7 (7.3–747.2)**	**344**	**125.5 (7.6–12,127.9)**	**461**	**44.9 (1.3–456.6)**	**341**	**38.6 (3.0–449.9)**

Footnote: Data derived from *P. falciparum* and *P. vivax* isolates fulfilling acceptability criteria (>66% rings at the start of the assay and assay duration ≥34 h).

Table 3
Allele frequencies of single nucleotide polymorphisms (SNPs) in *pfcrt* over time.

pfcrt		C72S	V73I	M74I	N75D/K	K76T	H97L/Y	T152A	S163R	A220S	Q271E	N326D/S	T333 A/S	I356 L/T	R371I
Year	n	Amino acid/Allele frequency													
2004	2	**S (1.0)**	V (1.0)	M (1.0)	N (1.0)	**T (1.0)**	H (1.0)	T (1.0)	S (1.0)	**S (1.0)**	Q (1.0)	**D (1.0)**	**A (1.0)**	**L (1.0)**	R (1.0)
2005	13	**S (1.0)**	V (1.0)	M (1.0)	N (1.0)	**T (1.0)**	H (1.0)	T (1.0)	S (1.0)	**S (1.0)**	Q (1.0)	**D (1.0)**	**A (1.0)**	**L (1.0)**	R (1.0)
2006	2	**S (1.0)**	V (1.0)	M (1.0)	N (1.0)	**T (1.0)**	H (1.0)	T (1.0)	S (1.0)	**S (1.0)**	Q (1.0)	**D (1.0)**	**A (1.0)**	**L (1.0)**	R (1.0)
2011	16	C (0.06) **S (0.94)**[a]	V (1.0)	M (1.0)	N (1.0)	K (0.06) **T (0.94)**[a]	H (1.0)	T (1.0)	S (1.0)	A (0.07) **S (0.94)**[a]	Q (1.0)	N (0.07) **D (0.94)**[a]	A (0.07) **S (0.94)**[a]	I (0.06) **T (0.94)**[a]	R (1.0)
2012	9	**S (1.0)**	V (1.0)	M (1.0)	N (1.0)	**T (1.0)**	H (1.0)	T (1.0)	S (1.0)	**S (1.0)**	Q (1.0)	**D (1.0)**	**A (1.0)**	**L (1.0)**	R (1.0)
2013	13	**S (1.0)**	V (1.0)	M (1.0)	N (1.0)	**T (1.0)**	H (1.0)	T (1.0)	S (1.0)	**S (1.0)**	Q (1.0)	**D (1.0)**	**A (1.0)**	**L (1.0)**	R (1.0)
2014	37	**S (1.0)**	V (1.0)	M (1.0)	N (1.0)	**T (1.0)**	H (1.0)	T (1.0)	S (1.0)	**S (1.0)**	Q (1.0)	**D (1.0)**	**A (1.0)**	**L (1.0)**	R (1.0)
2015	10	**S (1.0)**	V (1.0)	M (1.0)	N (1.0)	**T (1.0)**	H (1.0)	T (1.0)	S (1.0)	**S (1.0)**	Q (1.0)	**D (1.0)**	**A (1.0)**	**L (1.0)**	R (1.0)
2016	13	C (0.07) **S (0.93)**[b]	V (1.0)	M (1.0)	N (1.0)	K (0.07) **T (0.93)**[b]	H (1.0)	T (1.0)	S (1.0)	A (0.10) **S (0.93)**[b]	Q (1.0)	N (0.08) **D (0.93)**[b]	A (0.08) **S (0.93)**[b]	I (0.07) **T (0.93)**[b]	R (1.0)
2017	16	**S (1.0)**	V (1.0)	M (1.0)	N (1.0)	**T (1.0)**	H (1.0)	T (1.0)	S (1.0)	**S (1.0)**	Q (1.0)	**D (1.0)**	**A (1.0)**	**L (1.0)**	R (1.0)

[a] 95% confidence interval: 0.70–1.0.
[b] 95% confidence interval: 0.66–1.0.

reveals a bimodal drug responses for PIP resistant strains (Bopp et al., 2018) and thus, $IC_{90}s$ or a novel ring stage survival assay (RSA) have been proposed as better indicators of PIP resistance (Duru et al., 2015; Ross et al., 2018). PIP resistant isolates derived using gene-editing or *in vitro* selected laboratory strains under PIP pressure had $IC_{50}s > 200$ nM and $IC_{90}s > 1000$ nM (Parobek et al., 2017; Ross et al., 2018). In contrast, in our study the least susceptible Papuan isolate had a PIP IC_{50} of 221 nM ($IC_{90} = 450$ nM), with no evidence of a bimodal distribution in drug susceptibility.

Monitoring temporal drug susceptibility trends in clinical *Plasmodium* isolates is challenging particularly for *P. vivax* which cannot be sustained in *in vitro* culture. *Ex vivo* P. vivax drug susceptibility has to be conducted using fresh blood collected directly from infected patients often in remote malaria endemic settings with limited laboratory infrastructure. Furthermore, there is potential for significant sampling bias confounding the representativeness of the surveillance data. In our study, the isolates selected for *ex vivo* susceptibility analysis were restricted to those from patients presenting with parasitaemia between 2000 and 80,000 asexual parasites/μL and, for *P. vivax*, greater than 66% of parasites at ring stage at the start of the assay (Kerlin et al., 2012; Russell et al., 2008). Whilst application of these criteria limits the number of eligible isolates, they have been shown to improve the quality of the data, facilitating drug susceptibility testing of novel antimalarial compounds (Baragaña et al., 2015; Le Bihan et al., 2016; Marfurt et al., 2011b; Marfurt et al., 2012b; Nilsen et al., 2013; O'Neill et al., 2017; Phillips et al., 2016; Vaidya et al., 2014; Wirjanata et al., 2015b). The validity of the *P. vivax ex vivo* estimates of drug susceptibility is supported by their correlation with poor efficacy in clinical trials (Llanos-Cuentas et al., 2018; Phillips et al., 2016). However, an important limitation of schizont maturation testing is that assays for *P. vivax* isolates are constrained by inability to maintain cultures through schizont rupture and reinvasion. To explore the robustness of our findings a sensitivity analysis was conducted using tighter inclusion criteria (isolates with >90% rings and 34–42 h incubation as well as only isolates

Table 4
Allele frequencies of single nucleotide polymorphisms (SNPs) in *pfmdr*1 over time.

*pfmdr*1		N86Y		Y184F		S1034C	N1042D		D1246Y
Year	N	Amino acid/Allele frequency; 95% Confidence Intervals							
2004	35	N (0.66) **Y (0.34)**	0.21–0.51	Y (0.40) **F (0.60)**	0.44–0.74	S (1.0)	N (0.34) **D (0.66)**	0.49–0.79	D (1.0)
2005	46	N (0.80) **Y (0.20)**	0.10–0.33	Y (0.24) **F (0.76)**	0.62–0.86	S (1.0)	N (0.17) **D (0.83)**	0.69–0.91	D (1.0)
2006	8	N (0.63) **Y (0.37)**	0.13–0.70	Y (0.50) **F (0.50)**	0.22–0.78	S (1.0)	N (0.50) **D (0.50)**	0.22–0.78	D (1.0)
2011	16	N (0.94) **Y (0.06)**	0–0.30	Y (0.19) **F (0.81)**	0.56–0.94	S (1.0)	N (0.19) **D (0.81)**	0.56–0.94	D (1.0)
2012	9	N (1.0)		**F (1.0)**		S (1.0)	**D (1.0)**		D (1.0)
2013	13	N (0.92) **Y (0.08)**	0–0.35	N (0.08) **F (0.92)**	0.65–1.0	S (1.0)	N (0.08) **D (0.92)**	0.65–1.0	D (1.0)
2014	37	N (0.95) **Y (0.05)**	0.01–0.19	Y (0.08) **F (0.92)**	0.78–0.98	S (1.0)	N (0.08) **D (0.92)**	0.78–0.98	D (1.0)
2015	10	N (1.0)		**F (1.0)**		S (1.0)	**D (1.0)**		D (1.0)
2016	13	N (1.0)		N (0.14) **F (0.86)**	0.57–0.93	S (1.0)	N (0.14) **D (0.86)**	0.57–0.93	D (1.0)
2017	16	N (1.0)		Y (0.06) **F (0.94)**	0.70–1.0	S (1.0)	N (0.06) **D (0.94)**	0.70–1.0	D (1.0)

tested on drug plates with narrow quality control IC_{50s}). Reassuringly, the temporal trends for PIP remained the same, with no apparent temporal trend in CQ susceptibility.

Previous molecular analyses of clinical isolates of *P. falciparum* collected from a clinical efficacy trial of DHP between 2015 and 2016 showed none of the *kelch*13 polymorphisms that have been associated with artemisinin resistance (Ariey et al., 2014; Miotto et al., 2015; Poespoprodjo et al., 2018). In conjunction with rapid parasite clearance and cure rates exceeding 90%, this suggests that artemisinin resistance has yet to emerge in the province and the DHA component of the ACT is likely to continue reducing the selective PIP pressure on the parasite. In our current analysis, none of the known molecular markers of PIP resistance were detected in any isolate over the 14-year period.

In the GMS, *pfpm*2 copy number variants (CNV) (Amato et al., 2017; Witkowski et al., 2017) and *pfcrt* polymorphisms T93S, H97Y, C101F, F145I, I218F M343L, and G353V (Agrawal et al., 2017; Dhingra et al., 2017; Hamilton et al., 2019; Pelleau et al., 2015; Ross et al., 2018; van der Pluijm et al., 2019), and *exo*-E415G (Amato et al., 2017) occurred almost exclusively on a genetic artemisinin-resistant and the CQ-resistant Southeast-Asian Dd2-type *pfcrt* backgrounds. Indeed, transfection of *pfpm*2 CNVs on the CQ-sensitive 3D7-type *pfcrt* background did not result in PIP resistance *in vitro* (Loesbanluechai et al., 2019). Furthermore, characterization of *pfcrt* in field isolates from French Guiana, where CQ was withdrawn in 1995 and the prevalence of CQ resistant *P. falciparum* dropped from >90% to <30% in 2012, revealed persistence of the CQ-resistant South American 7G8-type *pfcrt* isoform, but an additional *pfcrt* mutation C350R that was associated with decreased PIP susceptibility *in vitro* (Pelleau et al., 2015).

PIP resistance-associated *pfcrt* SNPs in Dd2 and 7G8 strains are associated with PIP efflux from the digestive food vacuole, but reduced CQ transport (Riegel and Roepe, 2020). There was no inverse correlation, suggesting that the interactions between *pfcrt* and antimalarials are complex and most likely mediated by additional mutations in *pfcrt*, or genetic modifications in other genes. In Timika, the South American 7G8 *pfcrt* isoform has been fixed since 2005. As PIP resistance mutations come with a fitness cost and at the expense of losing the CQ-resistant phenotype (Dhingra et al., 2017; Riegel and Roepe, 2020; Ross et al., 2018), it appears that PIP pressure on the parasites remains low, presumably due to the high efficacy of DHA in reducing the exposed parasite biomass.

The temporal patterns of *pfmdr*1 SNPs in southern Papua show a different pattern than that of *pfcrt*. *Pfmdr*1 86Y, a modulator of CQ resistance (Foote et al., 1990), had a relatively low allele frequency (0.34) in 2005, despite very low CQ efficacy, whereas the frequencies of the 184F and 1042D polymorphisms were higher (0.60 and 0.66, respectively). Over the study period, 86Y allele frequencies decreased and became fixed at the wild-type allele in 2015, whereas the frequencies of 184F and 1042D increased and became almost fixed at the mutant alleles in 2017.

The role of PIP selective pressure on *pfmdr*1 is unclear. Molecular, clinical and *in vitro* phenotype association studies and allelic exchange experiments suggest different or no associations between SNPs or *pfmdr*1 amplification and PIP resistance (Gil and Krishna, 2017; Veiga et al., 2016). Amato and colleagues investigated the origin and spread of multidrug-resistant *P. falciparum* in South East Asia, and raised the possibility of 'natural antagonism' between *pfmdr*1 and *pfpm*2 amplification (Amato et al., 2017). Interestingly in Papua, Indonesia, no *pfmdr*1 CNVs were detected over the entire study period. In-depth analysis of 187 *P. falciparum* isolates for which *pfmdr*1 was genotyped (all on the *pfcrt* SVMNT background) showed that median CQ IC_{50}s did not differ between the seven observed genotypes, providing further evidence that *pfmdr*1 SNPs are secondary mediators of *P. falciparum* CQ resistance.

Since 2006, there was no temporal trend in the prevalence of putative molecular markers of CQ resistance in *P. vivax*, with polymorphisms in the *pvcrt-o* K10 and *pvmdr*1 967F and 1076L fixed at the mutant alleles, respectively. Hence, the large variation in *ex vivo* CQ susceptibility of *P. vivax* during the study period, in which IC_{50}s ranged from 4.6 to 855 nM, cannot be explained solely by currently proposed molecular markers of CQ resistance.

In conclusion, the *ex vivo* drug susceptibility and molecular analysis of CQ and PIP efficacy for *P. falciparum* and *P. vivax* after 12 years of intense drug pressure with DHP suggests that whilst the degree of CQ resistance appears to have been sustained, there has been a slight decline in PIP susceptibility, although this does not appear to have reached clinically significant levels. The observed decreasing trend in *ex vivo* PIP susceptibility highlights the importance of vigilance and ongoing monitoring.

Declaration of competing interest

None declared.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijpddr.2021.06.002.

References

Agrawal, S., Moser, K.A., Morton, L., Cummings, M.P., Parihar, A., Dwivedi, A., Shetty, A.C., Drabek, E.F., Jacob, C.G., Henrich, P.P., Parobek, C.M., Jongsakul, K., Huy, R., Spring, M.D., Lanteri, C.A., Chaorattanakawee, S., Lon, C., Fukuda, M.M., Saunders, D.L., Fidock, D.A., Lin, J.T., Juliano, J.J., Plowe, C.V., Silva, J.C., Takala-Harrison, S., 2017. Association of a novel mutation in the plasmodium falciparum chloroquine resistance transporter with decreased piperaquine sensitivity. J. Infect. Dis. 216, 468–476.

Amato, R., Lim, P., Miotto, O., Amaratunga, C., Dek, D., Pearson, R.D., Almagro-Garcia, J., Neal, A.T., Sreng, S., Suon, S., Drury, E., Jyothi, D., Stalker, J., Kwiatkowski, D.P., Fairhurst, R.M., 2017. Genetic markers associated with dihydroartemisinin-piperaquine failure in Plasmodium falciparum malaria in Cambodia: a genotype-phenotype association study. Lancet Infect. Dis. 17, 164–173.

Ariey, F., Witkowski, B., Amaratunga, C., Beghain, J., Langlois, A.C., Khim, N., Kim, S., Duru, V., Bouchier, C., Ma, L., Lim, P., Leang, R., Duong, S., Sreng, S., Suon, S., Chuor, C.M., Bout, D.M., Menard, S., Rogers, W.O., Genton, B., Fandeur, T., Miotto, O., Ringwald, P., Le, B.J., Berry, A., Barale, J.C., Fairhurst, R.M., Benoit-Vical, F., Mercereau-Puijalon, O., Menard, D., 2014. A molecular marker of artemisinin-resistant Plasmodium falciparum malaria. Nature 505, 50–55.

Auburn, S., Benavente, E.D., Miotto, O., Pearson, R.D., Amato, R., Grigg, M.J., Barber, B. E., William, T., Handayuni, I., Marfurt, J., Trimarsanto, H., Noviyanti, R., Sriprawat, K., Nosten, F., Campino, S., Clark, T.G., Anstey, N.M., Kwiatkowski, D.P., Price, R.N., 2018. Genomic analysis of a pre-elimination Malaysian Plasmodium vivax population reveals selective pressures and changing transmission dynamics. Nat. Commun. 9, 2585.

Auburn, S., Getachew, S., Pearson, R.D., Amato, R., Miotto, O., Trimarsanto, H., Zhu, S. J., Rumaseb, A., Marfurt, J., Noviyanti, R., Grigg, M.J., Barber, B., William, T., Goncalves, S.M., Drury, E., Sriprawat, K., Anstey, N.M., Nosten, F., Petros, B., Aseffa, A., McVean, G., Kwiatkowski, D.P., Price, R.N., 2019. Genomic analysis of plasmodium vivax in southern Ethiopia reveals selective pressures in multiple parasite mechanisms. J. Infect. Dis. 220, 1738–1749.

Baragaña, B., Hallyburton, I., Lee, M.C., Norcross, N.R., Grimaldi, R., Otto, T.D., Proto, W.R., Blagborough, A.M., Meister, S., Wirjanata, G., Ruecker, A., Upton, L.M., Abraham, T.S., Almeida, M.J., Pradhan, A., Porzelle, A., Luksch, T., Martínez, M.S., Luksch, T., Bolscher, J.M., Woodland, A., Norval, S., Zuccotto, F., Thomas, J., Simeons, F., Stojanovski, L., Osuna-Cabello, M., Brock, P.M., Churcher, T.S., Sala, K. A., Zakutansky, S.E., Jiménez-Díaz, M.B., Sanz, L.M., Riley, J., Basak, R., Campbell, M., Avery, V.M., Sauerwein, R.W., Dechering, K.J., Noviyanti, R., Campo, B., Frearson, J.A., Angulo-Barturen, I., Ferrer-Bazaga, S., Gamo, F.J., Wyatt, P.G., Leroy, D., Siegl, P., Delves, M.J., Kyle, D.E., Wittlin, S., Marfurt, J., Price, R.N., Sinden, R.E., Winzeler, E.A., Charman, S.A., Bebrevska, L., Gray, D.W., Campbell, S., Fairlamb, A.H., Willis, P.A., Rayner, J.C., Fidock, D.A., Read, K.D., Gilbert, I.H., 2015. A novel multiple-stage antimalarial agent that inhibits protein synthesis. Nature 522, 315–320.

Bopp, S., Magistrado, P., Wong, W., Schaffner, S.F., Mukherjee, A., Lim, P., Dhorda, M., Amaratunga, C., Woodrow, C.J., Ashley, E.A., White, N.J., Dondorp, A.M., Fairhurst, R.M., Ariey, F., Menard, D., Wirth, D.F., Volkman, S.K., 2018. Plasmepsin II-III copy number accounts for bimodal piperaquine resistance among Cambodian Plasmodium falciparum. Nat. Commun. 9, 1769.

Brunschwig, C., Lawrence, N., Taylor, D., Abay, E., Njoroge, M., Basarab, G.S., Le Manach, C., Paquet, T., Cabrera, D.G., Nchinda, A.T., de Kock, C., Wiesner, L., Denti, P., Waterson, D., Blasco, B., Leroy, D., Witty, M.J., Donini, C., Duffy, J., Wittlin, S., White, K.L., Charman, S.A., Jimenez-Diaz, M.B., Angulo-Barturen, I., Herreros, E., Gamo, F.J., Rochford, R., Mancama, D., Coetzer, T.L., van der Watt, M. E., Reader, J., Birkholtz, L.M., Marsh, K.C., Solapure, S.M., Burke, J.E., McPhail, J.A., Vanaerschot, M., Fidock, D.A., Fish, P.V., Siegl, P., Smith, D.A., Wirjanata, G., Noviyanti, R., Price, R.N., Marfurt, J., Silue, K.D., Street, L.J., Chibale, K., 2018. UCT943, a next-generation plasmodium falciparum PI4K inhibitor preclinical candidate for the treatment of malaria. Antimicrob. Agents Chemother. 62.

Cerutti, J.C., Marques, C., Alencar, F.E., Durlacher, R.R., Alween, A., Segurado, A.A., Pang, L.W., Zalis, M.G., 1999. Antimalarial drug susceptibility testing of Plasmodium falciparum in Brazil using a radioisotope method. Mem. Inst. Oswaldo Cruz 94, 803–809.

Chaorattanakawee, S., Saunders, D.L., Sea, D., Chanarat, N., Yingyuen, K., Sundrakes, S., Saingam, P., Buathong, N., Sriwichai, S., Chann, S., Se, Y., Yom, Y., Heng, T.K., Kong, N., Kuntawunginn, W., Tangthongchaiwiriya, K., Jacob, C., Takala-Harrison, S., Plowe, C., Lin, J.T., Chuor, C.M., Prom, S., Tyner, S.D., Gosi, P., Teja-Isavadharm, P., Lon, C., Lanteri, C.A., 2015. Ex vivo drug susceptibility testing and molecular profiling of clinical plasmodium falciparum isolates from Cambodia from 2008 to 2013 suggest emerging piperaquine resistance. Antimicrob. Agents Chemother. 59, 4631–4643.

Dhingra, S.K., Redhi, D., Combrinck, J.M., Yeo, T., Okombo, J., Henrich, P.P., Cowell, A. N., Gupta, P., Stegman, M.L., Hoke, J.M., Cooper, R.A., Winzeler, E., Mok, S., Egan, T.J., Fidock, D.A., 2017. A variant PfCRT isoform can contribute to plasmodium falciparum resistance to the first-line partner drug piperaquine. mBio 8.

Dondorp, A.M., Nosten, F., Yi, P., Das, D., Phyo, A.P., Tarning, J., Lwin, K.M., Ariey, F., Hanpithakpong, W., Lee, S.J., Ringwald, P., Silamut, K., Imwong, M., Chotivanich, K., Lim, P., Herdman, T., An, S.S., Yeung, S., Singhasivanon, P., Day, N. P., Lindegardh, N., Socheat, D., White, N.J., 2009. Artemisinin resistance in Plasmodium falciparum malaria. N. Engl. J. Med. 361, 455–467.

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

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, P.J., Huy, R., Mercereau-Puijalon, O., Witkowski, B., Menard, D., 2015. 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. BMC Med. 13, 305.

Fidock, D.A., Nomura, T., Talley, A.K., Cooper, R.A., Dzekunov, S.M., Ferdig, M.T., Ursos, L.M., Sidhu, A.B., Naude, B., Deitsch, K.W., Su, X.Z., Wootton, J.C., Roepe, P. D., Wellems, T.E., 2000. Mutations in the P. falciparum digestive vacuole transmembrane protein PfCRT and evidence for their role in chloroquine resistance. Mol. Cell. 6, 861–871.

Foote, S.J., Kyle, D.E., Martin, R.K., Oduola, A.M., Forsyth, K., Kemp, D.J., Cowman, A. F., 1990. Several alleles of the multidrug-resistance gene are closely linked to chloroquine resistance in Plasmodium falciparum. Nature 345, 255–258.

Frosch, A.E., Laufer, M.K., Mathanga, D.P., Takala-Harrison, S., Skarbinski, J., Claassen, C.W., Dzinjalamala, F.K., Plowe, C.V., 2014. Return of widespread chloroquine-sensitive Plasmodium falciparum to Malawi. J. Infect. Dis. 210, 1110–1114.

Gil, J.P., Krishna, S., 2017. pfmdr1 (Plasmodium falciparum multidrug drug resistance gene 1): a pivotal factor in malaria resistance to artemisinin combination therapies. Expert Rev. Anti Infect. Ther. 15, 527–543.

Hamilton, W.L., Amato, R., van der Pluijm, R.W., Jacob, C.G., Quang, H.H., Thuy-Nhien, N.T., Hien, T.T., Hongvanthong, B., Chindavongsa, K., Mayxay, M., Huy, R., Leang, R., Huch, C., Dysoley, L., Amaratunga, C., Suon, S., Fairhurst, R.M., Tripura, R., Peto, T.J., Sovann, Y., Jittamala, P., Hanboonkunupakarn, B., Pukrittayakamee, S., Chau, N.H., Imwong, M., Dhorda, M., Vongpromek, R., Chan, X.H.S., Maude, R.J., Pearson, R.D., Nguyen, T., Rockett, K., Drury, E., Gonçalves, S., White, N.J., Day, N.P., Kwiatkowski, D.P., Dondorp, A.M., Miotto, O., 2019. Evolution and expansion of multidrug-resistant malaria in southeast Asia: a genomic epidemiology study. Lancet Infect. Dis. 19, 943–951.

Hao, M., Jia, D., Li, Q., He, Y., Yuan, L., Xu, S., Chen, K., Wu, J., Shen, L., Sun, L., Zhao, H., Yang, Z., Cui, L., 2013. In vitro sensitivities of Plasmodium falciparum isolates from the China-Myanmar border to piperaquine and association with polymorphisms in candidate genes. Antimicrob. Agents Chemother. 57, 1723–1729.

Hayward, R., Saliba, K.J., Kirk, K., 2005. pfmdr1 mutations associated with chloroquine resistance incur a fitness cost in Plasmodium falciparum. Mol. Microbiol. 55, 1285–1295.

Kenangalem, E., Poespoprodjo, J.R., Douglas, N.M., Burdam, F.H., Gdeumana, K., Chalfein, F., Prayoga, Thio, F., Devine, A., Marfurt, J., Waramori, G., Yeung, S., Noviyanti, R., Penttinen, P., Bangs, M.J., Sugiarto, P., Simpson, J.A., Soenarto, Y., Anstey, N.M., Price, R.N., 2019. Malaria morbidity and mortality following introduction of a universal policy of artemisinin-based treatment for malaria in Papua, Indonesia: a longitudinal surveillance study. PLoS Med. 16, e1002815.

Kerlin, D.H., Boyce, K., Marfurt, J., Simpson, J.A., Kenangalem, E., Cheng, Q., Price, R. N., Gatton, M.L., 2012. An analytical method for assessing stage-specific drug activity in Plasmodium vivax malaria: implications for ex vivo drug susceptibility testing. PLoS Neglected Trop. Dis. 6, e1772.

Kuhen, K.L., Chatterjee, A.K., Rottmann, M., Gagaring, K., Borboa, R., Buenviaje, J., Chen, Z., Francek, C., Wu, T., Nagle, A., Barnes, S.W., Plouffe, D., Lee, M.C., Fidock, D.A., Graumans, W., van de Vegte-Bolmer, M., van Gemert, G.J., Wirjanata, G., Sebayang, B., Marfurt, J., Russell, B., Suwanarusk, R., Price, R.N., Nosten, F., Tungtaeng, A., Gettayacamin, M., Sattabongkot, J., Taylor, J., Walker, J. R., Tully, D., Patra, K.P., Flannery, E.L., Vinetz, J.M., Renia, L., Sauerwein, R.W., Winzeler, E.A., Glynne, R.J., Diagana, T.T., 2014. KAF156 is an antimalarial clinical

candidate with potential for use in prophylaxis, treatment, and prevention of disease transmission. Antimicrob. Agents Chemother. 58, 5060–5067.

Lambros, C., Vanderberg, J.P., 1979. Synchronization of Plasmodium falciparum erythrocytic stages in culture. J. Parasitol. 65, 418–420.

Le Bihan, A., de Kanter, R., Angulo-Barturen, I., Binkert, C., Boss, C., Brun, R., Brunner, R., Buchmann, S., Burrows, J., Dechering, K.J., Delves, M., Ewerling, S., Ferrer, S., Fischli, C., Gamo-Benito, F.J., Gnadig, N.F., Heidmann, B., Jimenez-Diaz, M.B., Leroy, D., Martinez, M.S., Meyer, S., Moehrle, J.J., Ng, C.L., Noviyanti, R., Ruecker, A., Sanz, L.M., Sauerwein, R.W., Scheurer, C., Schleiferboeck, S., Sinden, R., Snyder, C., Straimer, J., Wirjanata, G., Marfurt, J., Price, R.N., Weller, T., Fischli, W., Fidock, D.A., Clozel, M., Wittlin, S., 2016. Characterization of novel antimalarial compound ACT-451840: preclinical assessment of activity and dose-efficacy modeling. PLoS Med. 13, e1002138.

Llanos-Cuentas, A., Casapia, M., Chuquiyauri, R., Hinojosa, J.C., Kerr, N., Rosario, M., Toovey, S., Arch, R.H., Phillips, M.A., Rozenberg, F.D., Bath, J., Ng, C.L., Cowell, A. N., Winzeler, E.A., Fidock, D.A., Baker, M., Mohrle, J.J., Hooft van Huijsduijnen, R., Gobeau, N., Araeipour, N., Andenmatten, N., Ruckle, T., Duparc, S., 2018. Antimalarial activity of single-dose DSM265, a novel plasmodium dihydroorotate dehydrogenase inhibitor, in patients with uncomplicated Plasmodium falciparum or Plasmodium vivax malaria infection: a proof-of-concept, open-label, phase 2a study. Lancet Infect. Dis. 18, 874–883.

Loesbanluechai, D., Kotanan, N., de Cozar, C., Kochakarn, T., Ansbro, M.R., Chotivanich, K., White, N.J., Wilairat, P., Lee, M.C.S., Gamo, F.J., Sanz, L.M., Chookajorn, T., Kümpornsin, K., 2019. Overexpression of plasmepsin II and plasmepsin III does not directly cause reduction in Plasmodium falciparum sensitivity to artesunate, chloroquine and piperaquine. Int J Parasitol Drugs Drug Resist 9, 16–22.

Lourens, C., Watkins, W.M., Barnes, K.I., Sibley, C.H., Guerin, P.J., White, N.J., Lindegardh, N., 2010. Implementation of a reference standard and proficiency testing programme by the world wide antimalarial resistance network (WWARN). Malar. J. 9, 375.

Marfurt, J., de Monbrison, F., Brega, S., Barbollat, L., Muller, I., Sie, A., Goroti, M., Reeder, J.C., Beck, H.P., Picot, S., Genton, B., 2008. Molecular markers of in vivo Plasmodium vivax resistance to amodiaquine plus sulfadoxine-pyrimethamine: mutations in pvdhfr and pvmdr1. J. Infect. Dis. 198, 409–417.

Marfurt, J., Chalfein, F., Prayoga, P., Wabiser, F., Kenangalem, E., Piera, K.A., Fairlie, D. P., Tjitra, E., Anstey, N.M., Andrews, K.T., Price, R.N., 2011a. Ex vivo activity of histone deacetylase inhibitors against multidrug-resistant clinical isolates of Plasmodium falciparum and P. vivax. Antimicrob.Agents Chemother. 55, 961–966.

Marfurt, J., Chalfein, F., Prayoga, P., Wabiser, F., Kenangalem, E., Piera, K.A., Machunter, B., Tjitra, E., Anstey, N.M., Price, R.N., 2011b. Ex vivo drug susceptibility of ferroquine against chloroquine-resistant isolates of Plasmodium falciparum and P. vivax. Antimicrob.Agents Chemother. 55, 4461–4464.

Marfurt, J., Chalfein, F., Prayoga, P., Wabiser, F., Wirjanata, G., Sebayang, B., Piera, K.A., Wittlin, S., Haynes, R.K., Mohrle, J.J., Anstey, N.M., Kenangalem, E., Price, R.N., 2012a. Comparative ex vivo activity of novel endoperoxides in multidrug-resistant plasmodium falciparum and P. vivax. Antimicrob.Agents Chemother. 56, 5258–5263.

Marfurt, J., Chalfein, F., Prayoga, P., Wabiser, F., Wirjanata, G., Sebayang, B., Piera, K.A., Wittlin, S., Haynes, R.K., Mohrle, J.J., Anstey, N.M., Kenangalem, E., Price, R.N., 2012b. Comparative ex vivo activity of novel endoperoxides in multidrug-resistant plasmodium falciparum and P. vivax. Antimicrob. Agents Chemother. 56, 5258–5263.

McCallum, F.J., Birrell, G.W., Chavchich, M., Harris, I., , Obaldia, N. 3rd, Van Breda, K., Heffernan, G.D., Jacobus, D.P., Shanks, D., Edstein, M.D., 2020. In vivo efficacy and pharmacokinetics of the 2-aminomethylphenol antimalarial JPC-3210 in the Aotus monkey-human malaria model. Antimicrob. Agents Chemother. 64.

Miotto, O., Amato, R., Ashley, E.A., MacInnis, B., Almagro-Garcia, J., Amaratunga, C., Lim, P., Mead, D., Oyola, S.O., Dhorda, M., Imwong, M., Woodrow, C., Manske, M., Stalker, J., Drury, E., Campino, S., Amenga-Etego, L., Thanh, T.N., Tran, H.T., Ringwald, P., Bethell, D., Nosten, F., Phyo, A.P., Pukrittayakamee, S., Chotivanich, K., Chuor, C.M., Nguon, C., Suon, S., Sreng, S., Newton, P.N., Mayxay, M., Khanthavong, M., Hongvanthong, B., Htut, Y., Han, K.T., Kyaw, M.P., Faiz, M.A., Fanello, C.I., Onyamboko, M., Mokuolu, O.A., Jacob, C.G., Takala-Harrison, S., Plowe, C.V., Day, N.P., Dondorp, A.M., Spencer, C.C., McVean, G., Fairhurst, R.M., White, N.J., Kwiatkowski, D.P., 2015. Genetic architecture of artemisinin-resistant Plasmodium falciparum. Nat. Genet. 47, 226–234.

Mwai, L., Kiara, S.M., Abdirahman, A., Pole, L., Rippert, A., Diriye, A., Bull, P., Marsh, K., Borrmann, S., Nzila, A., 2009. In vitro activities of piperaquine, lumefantrine, and dihydroartemisinin in Kenyan Plasmodium falciparum isolates and polymorphisms in pfcrt and pfmdr1. Antimicrob. Agents Chemother. 53, 5069–5073.

Nilsen, A., LaCrue, A.N., White, K.L., Forquer, I.P., Cross, R.M., Marfurt, J., Mather, M. W., Delves, M.J., Shackleford, D.M., Saenz, F.E., Morrisey, J.M., Steuten, J., Mutka, T., Li, Y., Wirjanata, G., Ryan, E., Duffy, S., Kelly, J.X., Sebayang, B.F., Zeeman, A.M., Noviyanti, R., Sinden, R.E., Kocken, C.H.M., Price, R.N., Avery, V.M., Angulo-Barturen, I., Jimenez-Diaz, M.B., Ferrer, S., Herreros, E., Sanz, L.M., Gamo, F.J., Bathurst, I., Burrows, J.N., Siegl, P., Guy, R.K., Winter, R.W., Vaidya, A. B., Charman, S.A., Kyle, D.E., Manetsch, R., Riscoe, M.K., 2013. Quinolone-3-diarylethers: a new class of antimalarial drug. Sci. Transl. Med. 5, 177ra137.

Noedl, H., Socheat, D., Satimai, W., 2009. Artemisinin-resistant malaria in Asia. N. Engl. J. Med. 361, 540–541.

O'Neill, P.M., Amewu, R.K., Charman, S.A., Sabbani, S., Gnadig, N.F., Straimer, J., Fidock, D.A., Shore, E.R., Roberts, N.L., Wong, M.H., Hong, W.D., Pidathala, C., Riley, C., Murphy, B., Aljayyoussi, G., Gamo, F.J., Sanz, L., Rodrigues, J., Cortes, C. G., Herreros, E., Angulo-Barturen, I., Jimenez-Diaz, M.B., Bazaga, S.F., Martinez-Martinez, M.S., Campo, B., Sharma, R., Ryan, E., Shackleford, D.M., Campbell, S., Smith, D.A., Wirjanata, G., Noviyanti, R., Price, R.N., Marfurt, J., Palmer, M.J., Copple, I.M., Mercer, A.E., Ruecker, A., Delves, M.J., Sinden, R.E., Siegl, P., Davies, J., Rochford, R., Kocken, C.H.M., Zeeman, A.M., Nixon, G.L., Biagini, G.A., Ward, S.A., 2017. A tetraoxane-based antimalarial drug candidate that overcomes PfK13-C580Y dependent artemisinin resistance. Nat. Commun. 8, 15159.

Parobek, C.M., Parr, J.B., Brazeau, N.F., Lon, C., Chaorattanakawee, S., Gosi, P., Barnett, E.J., Norris, L.D., Meshnick, S.R., Spring, M.D., Lanteri, C.A., Bailey, J.A., Saunders, D.L., Lin, J.T., Juliano, J.J., 2017. Partner-drug resistance and population substructuring of artemisinin-resistant plasmodium falciparum in Cambodia. Genome Biol Evol 9, 1673–1686.

Pearson, R.D., Amato, R., Auburn, S., Miotto, O., Almagro-Garcia, J., Amaratunga, C., Suon, S., Mao, S., Noviyanti, R., Trimarsanto, H., Marfurt, J., Anstey, N.M., William, T., Boni, M.F., Dolecek, C., Hien, T.T., White, N.J., Michon, P., Siba, P., Tavul, L., Harrison, G., Barry, A., Mueller, I., Ferreira, M.U., Karunaweera, N., Randrianarivelojosia, M., Gao, Q., Hubbart, C., Hart, L., Jeffery, B., Drury, E., Mead, D., Kekre, M., Campino, S., Manske, M., Cornelius, V.J., MacInnis, B., Rockett, K.A., Miles, A., Rayner, J.C., Fairhurst, R.M., Nosten, F., Price, R.N., Kwiatkowski, D.P., 2016. Genomic analysis of local variation and recent evolution in Plasmodium vivax. Nat. Genet. 48, 959–964.

Pearson, R.D., Amato, R., Kwiatkowski, D.P., Project, M.P.f.C., 2021 Feb 24. An open dataset of Plasmodium falciparum genome variation in 7,000 worldwide samples. Wellcome Open Res. 6 (42) https://doi.org/10.12688/wellcomeopenres.16168.1. eCollection.2021. Ambroise Ahouidi [1], Mozam Ali [2], Jacob Almagro-Garcia [2 3], Alfred Amambua-Ngwa [2 4], Chanaki Amaratunga [5], Roberto Amato [2 3], Lucas Amenga-Etego [6 7], Ben Andagalu [8], Tim J C Anderson [9], Voahangy Andrianaranjaka [10], Tobias Apinjoh [11], Cristina Ariani [2], Elizabeth A Ashley [12], Sarah Auburn [13 14], Gordon Awandare [7 15], Hampate Ba [16], Vito Baraka [17 18], Alyssa E Barry [19 20 21], Philip Bejon [22], Gwladys I Bertin [23], Maciej F Boni [14 24], Steffen Borrmann [25], Teun Bousema [26 27], Oralee Branch [28], Peter C Bull [22 29], George B J Busby [3], Thanat Chookajorn [30], Kesinee Chotivanich [30], Antoine Claessens [4 31], David Conway [26], Alister Craig [32 33], Umberto D'Alessandro [4], Souleymane Dama [34], Nicholas Pj Day [12], Brigitte Denis [33], Mahamadou Diakite [34], Abdoulaye Djimdé [34], Christiane Dolecek [14], Arjen M Dondorp [12], Chris Drakeley [26], Eleanor Drury [2], Patrick Duffy [5], Diego F Echeverry [35 36], Thomas G Egwang [37], Berhanu Erko [38], Rick M Fairhurst [39], Abdul Faiz [40], Caterina A Fanello [12], Mark M Fukuda [41], Dionicia Gamboa [42], Anita Ghansah [43], Lemu Golassa [38], Sonia Goncalves [2], William L Hamilton [2 44], G L Abby Harrison [21], Lee Hart [3], Christa Henrichs [3], Tran Tinh Hien [24 45], Catherine A Hill [46], Abraham Hodgson [47], Christina Hubbart [48], Mallika Imwong [30], Deus S Ishengoma [17 49], Scott A Jackson [50], Chris G Jacob [2], Ben Jeffery [3], Anna E Jeffreys [48], Kimberly J Johnson [3], Dushyanth Jyothi [2], Claire Kamaliddin [23], Edwin Kamau [51], Mihir Kekre [2], Krzysztof Kluczynski [3], Theerarat Kochakarn [2 30], Abibatou Konaté [52], Dominic P Kwiatkowski [2 3 48], Myat Phone Kyaw [53 54], Pharath Lim [5 55], Chanthap Lon [41], Kovana M Loua [56], Oumou Maïga-Ascofaré [34 57 58], Cinzia Malangone [2], Magnus Manske [2], Jutta Marfurt [13], Kevin Marsh [14 59], Mayfong Mayxay [60 61], Alistair Miles [2 3], Olivo Miotto [2 3 12], Victor Mobegi [62], Olugbenga A Mokuolu [63], Jacqui Montgomery [64], Ivo Mueller [21 65], Paul N Newton [66], Thuy Nguyen [2], Thuy-Nhien Nguyen [24], Harald Noedl [67], Francois Nosten [14 68], Rintis Noviyanti [69], Alexis Nzila [70], Lynette I Ochola-Oyier [22], Harold Ocholla [71 72], Abraham Oduro [6], Irene Omedo [22], Marie A Onyamboko [73], Jean-Bosco Ouedraogo [74], Kolapo Oyebola [75 76], Richard D Pearson [2 3], Norbert Peshu [22], Aung Pyae Phyo [12 68], Chris V Plowe [77], Ric N Price [12 13 45], Sasithon Pukrittayakamee [30], Milijaona Randrianarivelojosia [78 79], Julian C Rayner [2], Pascal Ringwald [80], Kirk A Rockett [2 48], Katherine Rowlands [48], Lastenia Ruiz [81], David Saunders [41], Alex Shayo [82], Peter Siba [83], Victoria J Simpson [3], Jim Stalker [2], Xin-Zhuan Su [5], Colin Sutherland [26], Shannon Takala-Harrison [84], Livingstone Tavul [83], Vandana Thathy [22 85], Antoinette Tshefu [86], Federica Verra [87], Joseph Vinetz [42 88], Thomas E Wellems [5], Jason Wendler [48], Nicholas J White [12], Ian Wright [3], William Yavo [52 89], Htut Ye [90].

Pelleau, S., Moss, E.L., Dhingra, S.K., Volney, B., Casteras, J., Gabryszewski, S.J., Volkman, S.K., Wirth, D.F., Legrand, E., Fidock, D.A., Neafsey, D.E., Musset, L., 2015. Adaptive evolution of malaria parasites in French Guiana: reversal of chloroquine resistance by acquisition of a mutation in pfcrt. Proc. Natl. Acad. Sci. U. S.A. 112, 11672–11677.

Phillips, M.A., White, K.L., Kokkonda, S., Deng, X., White, J., El Mazouni, F., Marsh, K., Tomchick, D.R., Manjalanagara, K., Rudra, K.R., Wirjanata, G., Noviyanti, R., Price, R.N., Marfurt, J., Shackleford, D.M., Chiu, F.C., Campbell, M., Jimenez-Diaz, M.B., Bazaga, S.F., Angulo-Barturen, I., Martinez, M.S., Lafuente-Monasterio, M., Kaminsky, W., Silue, K., Zeeman, A.M., Kocken, C., Leroy, D., Blasco, B., Rossignol, E., Rueckle, T., Matthews, D., Burrows, J.N., Waterson, D., Palmer, M.J., Rathod, P.K., Charman, S.A., 2016. A triazolopyrimidine-based dihydroorotate dehydrogenase inhibitor with improved drug-like properties for treatment and prevention of malaria. ACS Infect. Dis. 2, 945–957.

Poespoprodjo, J.R., Kenangalem, E., Wafom, J., Chandrawati, F., Puspitasari, A.M., Ley, B., Trianty, L., Korten, Z., Surya, A., Syafruddin, D., Anstey, N.M., Marfurt, J., Noviyanti, R., Price, R.N., 2018. Therapeutic response to dihydroartemisinin-piperaquine for P. Falciparum and P. Vivax nine years after its introduction in southern Papua, Indonesia. Am. J. Trop. Med. Hyg. 98, 677–682.

Popovici, J., Ménard, D., 2015. Challenges in antimalarial drug treatment for vivax malaria control. Trends Mol. Med. 21, 776–788.

Price, R.N., Marfurt, J., Chalfein, F., Kenangalem, E., Piera, K.A., Tjitra, E., Anstey, N.M., Russell, B., 2010. In vitro activity of pyronaridine against multidrug-resistant Plasmodium falciparum and Plasmodium vivax. Antimicrob. Agents Chemother. 54, 5146–5150.

Ratcliff, A., Siswantoro, H., Kenangalem, E., Wuwung, M., Brockman, A., Edstein, M.D., Laihad, F., Ebsworth, E.P., Anstey, N.M., Tjitra, E., Price, R.N., 2007. Therapeutic response of multidrug-resistant Plasmodium falciparum and P. vivax to chloroquine

and sulfadoxine-pyrimethamine in southern Papua, Indonesia. Trans. R. Soc. Trop. Med. Hyg. 101, 351–359.

Richards, W.H., Williams, S.G., 1973. The removal of leucocytes from malaria infected blood. Ann. Trop. Med. Parasitol. 67, 249–250.

Riegel, B., Roepe, P.D., 2020. Altered drug transport by plasmodium falciparum chloroquine resistance transporter isoforms harboring mutations associated with piperaquine resistance. Biochemistry 59, 2484–2493.

Ross, L.S., Dhingra, S.K., Mok, S., Yeo, T., Wicht, K.J., Kümpornsin, K., Takala-Harrison, S., Witkowski, B., Fairhurst, R.M., Ariey, F., Menard, D., Fidock, D.A., 2018. Emerging Southeast Asian PfCRT mutations confer Plasmodium falciparum resistance to the first-line antimalarial piperaquine. Nat. Commun. 9, 3314.

Russell, B., Chalfein, F., Prasetyorini, B., Kenangalem, E., Piera, K., Suwanarusk, R., Brockman, A., Prayoga, P., Sugiarto, P., Cheng, Q., Tjitra, E., Anstey, N.M., Price, R. N., 2008. Determinants of in vitro drug susceptibility testing of Plasmodium vivax. Antimicrob. Agents Chemother. 52, 1040–1045.

Sa, J.M., Yamamoto, M.M., Fernandez-Becerra, C., de Azevedo, M.F., Papakrivos, J., Naude, B., Wellems, T.E., Del Portillo, H.A., 2006. Expression and function of pvcrt-o, a Plasmodium vivax ortholog of pfcrt. In: Plasmodium Falciparum and Dictyostelium discoideum. Molecular and Biochemical Parasitology, vol. 150, pp. 219–228.

Saunders, D.L., Vanachayangkul, P., Lon, C., , Program, U.S.A.M.M.R., National Center for Parasitology, E., Malaria, C., Royal Cambodian Armed, F., 2014. Dihydroartemisinin-piperaquine failure in Cambodia. N. Engl. J. Med. 371, 484–485.

Suwanarusk, R., Russell, B., Chavchich, M., Chalfein, F., Kenangalem, E., Kosaisavee, V., Prasetyorini, B., Piera, K.A., Barends, M., Brockman, A., Lek-Uthai, U., Anstey, N.M., Tjitra, E., Nosten, F., Cheng, Q., Price, R.N., 2007. Chloroquine resistant Plasmodium vivax: in vitro characterisation and association with molecular polymorphisms. PloS One 2, e1089.

Thomson-Luque, R., Adams, J.H., Kocken, C.H.M., Pasini, E.M., 2019. From marginal to essential: the golden thread between nutrient sensing, medium composition and Plasmodium vivax maturation in in vitro culture. Malar. J. 18, 344, 344.

Trager, W., Jensen, J.B., 1976. Human malaria parasites in continuous culture. Science 193, 673–675.

Vaidya, A.B., Morrisey, J.M., Zhang, Z., Das, S., Daly, T.M., Otto, T.D., Spillman, N.J., Wyvratt, M., Siegl, P., Marfurt, J., Wirjanata, G., Sebayang, B.F., Price, R.N., Chatterjee, A., Nagle, A., Stasiak, M., Charman, S.A., Angulo-Barturen, I., Ferrer, S., Belen Jimenez-Diaz, M., Martinez, M.S., Gamo, F.J., Avery, V.M., Ruecker, A., Delves, M., Kirk, K., Berriman, M., Kortagere, S., Burrows, J., Fan, E., Bergman, L.W., 2014. Pyrazoleamide compounds are potent antimalarials that target Na+ homeostasis in intraerythrocytic Plasmodium falciparum. Nat. Commun. 5, 5521.

van der Pluijm, R.W., Imwong, M., Chau, N.H., Hoa, N.T., Thuy-Nhien, N.T., Thanh, N. V., Jittamala, P., Hanboonkunupakarn, B., Chutasmit, K., Saelow, C., Runjarern, R., Kaewmok, W., Tripura, R., Peto, T.J., Yok, S., Suon, S., Sreng, S., Mao, S., Oun, S., Yen, S., Amaratunga, C., Lek, D., Huy, R., Dhorda, M., Chotivanich, K., Ashley, E.A., Mukaka, M., Waithira, N., Cheah, P.Y., Maude, R.J., Amato, R., Pearson, R.D., Gonçalves, S., Jacob, C.G., Hamilton, W.L., Fairhurst, R.M., Tarning, J., Winterberg, M., Kwiatkowski, D.P., Pukrittayakamee, S., Hien, T.T., Day, N.P., Miotto, O., White, N.J., Dondorp, A.M., 2019. Determinants of dihydroartemisinin-piperaquine treatment failure in Plasmodium falciparum malaria in Cambodia, Thailand, and Vietnam: a prospective clinical, pharmacological, and genetic study. Lancet Infect. Dis. 19, 952–961.

Veiga, M.I., Dhingra, S.K., Henrich, P.P., Straimer, J., Gnädig, N., Uhlemann, A.C., Martin, R.E., Lehane, A.M., Fidock, D.A., 2016. Globally prevalent PfMDR1 mutations modulate Plasmodium falciparum susceptibility to artemisinin-based combination therapies. Nat. Commun. 7, 11553.

Wirjanata, G., Sebayang, B.F., Chalfein, F., Prayoga, Handayuni, I., Noviyanti, R., Kenangalem, E., Poespoprodjo, J.R., Burgess, S.J., Peyton, D.H., Price, R.N., Marfurt, J., 2015a. Contrasting ex vivo efficacies of "reversed chloroquine" compounds in chloroquine-resistant Plasmodium falciparum and P. vivax isolates. Antimicrob. Agents Chemother. 59, 5721–5726.

Wirjanata, G., Sebayang, B.F., Chalfein, F., Prayoga, Handayuni, I., Trianty, L., Kenangalem, E., Noviyanti, R., Campo, B., Poespoprodjo, J.R., Mohrle, J.J., Price, R. N., Marfurt, J., 2015b. Potent ex vivo activity of naphthoquine and methylene blue against drug-resistant clinical isolates of plasmodium falciparum and plasmodium vivax. Antimicrob. Agents Chemother. 59, 6117–6124.

Wirjanata, G., Handayuni, I., Prayoga, P., Leonardo, L., Apriyanti, D., Trianty, L., Wandosa, R., Gobay, B., Kenangalem, E., Poespoprodjo, J.R., Noviyanti, R., Kyle, D. E., Cheng, Q., Price, R.N., Marfurt, J., 2017. Plasmodium falciparum and P. vivax demonstrate contrasting chloroquine resistance reversal phenotypes. Antimicrob. Agents Chemother. 61 (8), e00355-17 https://doi.org/10.1128/AAC.00355-17.

Witkowski, B., Duru, V., Khim, N., Ross, L.S., Saintpierre, B., Beghain, J., Chy, S., Kim, S., Ke, S., Kloeung, N., Eam, R., Khean, C., Ken, M., Loch, K., Bouillon, A., Domergue, A., Ma, L., Bouchier, C., Leang, R., Huy, R., Nuel, G., Barale, J.C., Legrand, E., Ringwald, P., Fidock, D.A., Mercereau-Puijalon, O., Ariey, F., Ménard, D., 2017. A surrogate marker of piperaquine-resistant Plasmodium falciparum malaria: a phenotype-genotype association study. Lancet Infect. Dis. 17, 174–183.

Wongsrichanalai, C., Pickard, A.L., Wernsdorfer, W.H., Meshnick, S.R., 2002. Epidemiology of drug-resistant malaria. Lancet Infect. Dis. 2, 209–218.

Woodrow, C.J., Dahlstrom, S., Cooksey, R., Flegg, J.A., Le, N.H., Mentre, F., Murillo, C., Menard, D., Nosten, F., Sriprawat, K., Musset, L., Quashie, N.B., Lim, P., Fairhurst, R. M., Nsobya, S.L., Sinou, V., Noedl, H., Pradines, B., Johnson, J.D., Guerin, P.J., Sibley, C.H., Le, B.J., 2013. High-throughput analysis of antimalarial susceptibility data by the WorldWide Antimalarial Resistance Network (WWARN) in vitro analysis and reporting tool. Antimicrob. Agents Chemother. 57, 3121–3130.

World Health Organization (WHO), 2019. World Malaria Report 2019. World Health Organization, Geneva, Switzerland.

Xue, L., Shi, D.H., Harjani, J.R., Huang, F., Beveridge, J.G., Dingjan, T., Ban, K., Diab, S., Duffy, S., Lucantoni, L., Fletcher, S., Chiu, F.C.K., Blundell, S., Ellis, K., Ralph, S.A., Wirjanata, G., Teguh, S., Noviyanti, R., Chavchich, M., Creek, D., Price, R.N., Marfurt, J., Charman, S.A., Cuellar, M.E., Strasser, J.M., Dahlin, J.L., Walters, M.A., Edstein, M.D., Avery, V.M., Baell, J.B., 2019. 3,3'-Disubstituted 5,5'-Bi(1,2,4-triazine) derivatives with potent in vitro and in vivo antimalarial activity. J. Med. Chem. 62, 2485–2498.