Plasmodium falciparum isolates: *ex vivo* drug response

Edem Adika [1]†, Samirah Saiid[1], Enock K. Amoako[1], Mona-Liza E. Sakyi[1], Ahmed Osumanu[1], Collins Morang'a[1], Daniel Dosoo[1], Jersley D. Chirawurah[1], Gordon A. Awandare[1,2], Alfred Amambua-Ngwa [3], Yaw Aniweh[1] and Lucas N. Amenga-Etego[1]*

[1]*West African Centre for Cell Biology of Infectious Pathogens (WACCBIP), University of Ghana, Accra, Ghana;*
[2]*Department of Biochemistry, Cell and Molecular Biology (BCMB), University of Ghana, Accra, Ghana;* [3]*Medical Research Council Unit, The Gambia at LSHTM, Banjul, The Gambia*

*Corresponding author. E-mail: lamengaetego@ug.edu.gh
†Present address: Biological Chemistry and Drug Discovery (BCDD), School of Life Sciences (SLS), University of Dundee, Dundee, UK
𝕏 @eddy_may4, @LucasAmenga_Ete

Objectives: While artemisinin-based combination therapies (ACTs) are effective in sub-Saharan Africa, clinical isolates that are refractory to artemisinin derivatives are emerging in East Africa and ACT partner drugs are becoming less effective in West Africa. We investigated the *ex vivo* responses of *Plasmodium falciparum* clinical isolates to frontline antimalarials and the contribution of validated molecular markers of antimalarial drug resistance.

Methods: *Ex vivo* susceptibility was measured for 66 clinical isolates collected from uncomplicated malaria patients. IC_{50} was measured for dihydroartemisinin, artesunate, lumefantrine, amodiaquine and chloroquine using a SYBR Green I growth inhibition assay. We also assessed known drug resistance-mediating polymorphisms in *pfcrt*, *pfmdr1* and *pfkelch13* using Oxford Nanopore amplicon sequencing.

Results: *P. falciparum* clinical isolates were susceptible to dihydroartemisinin and artesunate. Clinical isolates showed a wide distribution of susceptibility to lumefantrine and amodiaquine, with some parasites having IC_{50} values above reference cut-offs for resistance to lumefantrine (150 nM) and amodiaquine (60 nM), suggesting decreased drug susceptibility. Ninety-seven percent of the isolates carried WT *pfcrt* K76 and *pfmdr1* N86 alleles, reported to mediate reduced response to lumefantrine and artemether/lumefantrine. *pfmdr1* N86 and 184F haplotype was carried by 62.1% of parasites. None of the clinical isolates carried validated *pfkelch13* mutations known to mediate artemisinin partial resistance.

Conclusions: Clinical isolates from coastal Ghana remain susceptible to artemisinin derivatives in commonly used ACTs in Ghana. However, we observed lower susceptibility to the ACT partner drugs lumefantrine and amodiaquine, suggesting the emergence of drug-tolerance phenotypes. Consistent surveillance of drug phenotype–genotype is needed to support ACT efficacy in Ghana.

Introduction

The gains made in malaria control since 2016 had plateaued just before the COVID-19 pandemic, but post-COVID-19 estimates suggest a worsening burden of malaria, particularly in the WHO African region.[1] There were an estimated 249 million cases and 608 000 deaths globally in 2022, despite considerable malaria control efforts.[1] Besides the disruptions in malaria control interventions imposed by the pandemic, a key factor in the historical resurgence of malaria is the rapid development of resistance in *Plasmodium falciparum* to frontline antimalarials.[2]

Multiple independent emergence and spread of artemisinin resistance in Southeast Asia (SEA) contributed to treatment failures with artemisinin-based combination therapies (ACT).[3–6] Africa faces the dual risk of the spread of artemisinin resistance from SEA to the continent and/or independent emergence of artemisinin resistance. There is growing evidence of emerging partial artemisinin resistance in East Africa with recent reports

in Rwanda, Uganda, and Tanzania, and the Horn of Africa (in Ethiopia and Eritrea).[7–10] This, in combination with growing reports of ACT partner drug tolerance across sub-Saharan Africa (sSA), poses risks to malaria control and elimination efforts. This calls for active surveillance of antimalarial drug efficacy against parasite populations, particularly in West Africa where transmission and drug pressure remain highest.[11,12]

Therapeutic efficacy studies (TESs) are the gold standard for monitoring the efficacy of antimalarial treatments but are not routinely feasible due to huge financial and logistic constraints.[13] *Ex vivo* and *in vitro* drug susceptibility studies offer an alternative approach of generating complementary data for assessing parasite responses to drugs outside the human host. These approaches, though with limitations, provide valuable information on parasite drug response patterns when combined with the study of molecular markers, which are potentially unbiased.[13,14]

Antimalarial drug policy in Ghana changed in 2005 from chloroquine to ACTs, in compliance with WHO recommendations.[15] Artemether/lumefantrine and artesunate/amodiaquine are now the frontline alternative treatments for uncomplicated malaria.[15] More recently, dihydroartemisinin/piperaquine was introduced as a third treatment option.[16] Sulfadoxine/pyrimethamine remains the mainstay for malaria prophylaxis during pregnancy and combined with amodiaquine for seasonal malaria chemoprevention (SMC) in children under 5 years in the northern Sahelian belt, where malaria transmission is seasonal.[17] These drug interventions together exert substantial selection pressure on *P. falciparum* populations, which may result in parasite adaptation and variable responses to frontline drugs.

We have previously demonstrated the utility of Oxford Nanopore Technology (ONT) sequencing for malaria molecular surveillance in resource-poor settings.[18] Here, we determined whether the susceptibility of clinical isolates has been affected by using multiple frontline ACTs, and employed an ONT-based amplicon sequencing panel to assess the prevalence of genetic polymorphisms in known antimalarial drug resistance loci. These included *P. falciparum* kelch13 (*pfkelch13*) for artemisinin, *P. falciparum* MDR gene 1 (*pfmdr1*) for amodiaquine and lumefantrine, and *P. falciparum* chloroquine resistance transporter (*pfcrt*) for chloroquine. The association of known markers of antimalarial drug resistance with *ex vivo* drug susceptibility is critical for identifying markers that correlate with phenotypic resistance as part of monitoring drug responses of Ghanaian parasites to artemisinin derivatives and ACT partner drugs.

Methods

Study site and procedure

The study was conducted at the Ledzokuku-Krowor Municipal Assembly (LEKMA) Hospital, located at Teshie in the Greater Accra Region (Figure S1, available as Supplementary data at *JAC* Online) of Ghana. Before the study, ethical approval was obtained from the Ethics Committee for Basic and Applied Sciences (ECBAS), College of Basic and Applied Sciences, University of Ghana (Ethics Reference Number: ECBAS 030/21-22). All study participants or their guardians gave written informed consent and additional assent was obtained for children aged between 12 and 17 years.

Individuals of all ages who presented with acute fever (or a history of fever within the past 24 h) were screened with the Combo malaria rapid diagnostic test (mRDT) based on *P. falciparum* histidine-rich protein 2

(HRP2) and *Plasmodium* lactate dehydrogenase (pLDH). Individuals who were on antimalarials or had taken any antimalarials within the past 2 weeks, and individuals with mixed-species infection, mostly *P. falciparum* and *P. malariae*, were excluded. Five millilitres of venous blood was collected from each participant into EDTA tubes and transported in a cool box to the West African Centre for Cell Biology of Infectious Pathogens (WACCBIP), University of Ghana for processing and analyses (see further details in the Supplementary data).

Ex vivo *growth inhibition drug assay*

The SYBR Green I-based antimalarial drug assay was set up as previously described, with few modifications.[19] Ninety microlitres of processed infected RBCs in complete media without serum (0.5% parasitaemia, 2% haematocrit) from the freshly collected clinical samples was added to 10 μL of serially diluted dihydroartemisinin (50–0.195 nM), artesunate (150–0.586 nM), lumefantrine (750–2.930 nM), amodiaquine (100–0.391 nM), chloroquine (750–2.930 nM) and complete media (no drug control) in 96-well plates. Three technical replicate assays were set up for each antimalarial compound. Drug assay plates were gassed (2% O_2 and 5% CO_2 balanced with N_2) in a modular incubator chamber and incubated for 72 h at 37°C. 2× SYBR Green I (Invitrogen, USA) in lysis buffer [20 mM Tris (pH 7.5), 5 mM EDTA, 0.008% (w/v) saponin and 0.08% (v/v) Triton X-100] was prepared by mixing 15 μL of SYBR Green I nucleic acid stain (Invitrogen, USA) with 45 mL of sterile lysis buffer. Assay plates were lysed with 1× final concentration SYBR Green I lysis buffer, incubated in the dark for 1 h and read on a Varioskan LUX multimode microplate reader (Thermo Fisher Scientific, USA) at an excitation wavelength of 485 nm and an emission wavelength of 520 nm. The half-maximal inhibitory concentrations (IC_{50}s) were estimated from the dose–response data for each antimalarial compound using non-linear regression analysis in GraphPad Prism (version 9.0, GraphPad Software, San Diego, CA, USA).

Genotyping of validated drug resistance-associated mutations in P. falciparum

Genomic DNA (gDNA) was extracted from aliquots of the processed infected RBCs using the QIAamp® DNA Blood Mini Kit (QIAGEN, Germany) following the manufacturer's protocol. Purified gDNA was quantified using the Invitrogen Qubit™ 1× dsDNA High Sensitivity (HS) assay kit with a Qubit fluorometer (Thermo Fisher Scientific, USA) and stored at −20°C. Amplicons of *pfcrt*, *pfdhfr*, *pfdhps*, *pfmdr1* and *pfkelch13* were generated by multiplex PCR as previously described.[18] Nuclease-free water (negative control) and gDNA of the *P. falciparum* KH02 isolate (positive control) were included in each PCR run. PCR was run on the PCRmax™ Alpha Cycler 4 (Thermo Fisher Scientific, USA) under preset thermocycling conditions.[18] A 2% agarose gel was run and visualized to confirm amplicon sizes.

Amplicon purification and library preparation

PCR amplicons were purified using the QIAamp® DNA MinElute Kit (QIAGEN, Germany) according to the manufacturer's instructions. Eluted DNA amplicons were quantified and stored at 4°C. Three sequencing libraries were prepared for the purified PCR amplicons (22 clinical isolates, KH02 positive control and negative control per batch) using the SQK-NBD112.24 native barcoding kit (ONT, UK). The manufacturer's protocol was followed for the library preparation. Briefly, 12.5 μL of each purified DNA amplicon (∼200 fmol) was end-prepped using 1× Ultra II End-prep enzyme mix incubated for 5 min at 20°C and 5 min at 65°C. End-prepped amplicons were purified with 1× AMPure XP Beads, eluted in 10 μL nuclease-free water and quantified.

Purified end-prepped DNA was barcoded with 24 unique native barcodes using 1× Blunt/TA Ligase master mix for 20 min at room temperature (RT). After barcoding, all 24 samples were pooled and purified with

1× AMPure XP. Pooled barcoded DNA amplicons were eluted in 35 µL of nuclease-free water and quantified. Pooled barcoded DNA (30 µL) was ligated to the Adapter Mix II H (AMII H) using the Quick T4 Ligase for 20 min at RT. Purification was performed with 1× AMPure XP using short fragment buffer (SFB). Adapter-ligated amplicons were eluted in 15 µL of elution buffer and quantified.

Sequencing, base-calling, alignment and SNP detection

DNA libraries for sequencing were prepared by thoroughly mixing 12 µL of prepared DNA library (~20 fmol), 37.5 µL of sequencing buffer II (SBII) and 25.5 µL of loading beads II (LBII). Seventy-five microlitres of the mixture was gently administered to flow cells (version FLO-MIN10.7) in the MinION Mk1b sequencer (MN39679). Sequencing was performed between 6 and 8 h with real-time high-accuracy *guppy* base calling (version 7.0.8) using the MinKNOW software (version 23.07.8). The resulting fastq files were processed through a custom Nextflow pipeline: *nano-rave* (Nanopore Rapid Analysis and Variant Explorer).[17] After quality control (QC) checks, sequence reads were mapped to 3D7 reference sequences for each amplicon target genes using *minimap2*. Amplicon coverage data was generated using *BEDTools*. *Medaka haploid* was used for variant calling to generate variant call format (VCF) file outputs for each amplicon for each sample (ONT barcode). VCF files were processed using custom R scripts (RStudio software, version 4.1.2) to calculate SNP frequencies at each drug resistance locus. A cut-off of >50× coverage was applied for an amplicon to be included in the analysis. All 66 *P. falciparum* clinical isolates collected were successfully sequenced (Figure S2).

Sequence alignment and phylogenetic analysis

A custom bash script was used to extract the 3D7 (PlasmoDB release 9.0) reference sequences for each sequenced resistance gene. These were indexed using samtools faidx, and sequence dictionaries were created with Picard CreateSequenceDictionary. BED files containing the chromosome bounds were generated from the *samtools fai* indexes. These BED files were converted to Picard interval lists using *BedToIntervalList*. The *Expasy* online tool (https://web.expasy.org/translate/) was used to translate all the consensus sequences for resistance genes in FASTA format to amino acid sequences for the 3D7 reference, Dd2 and clinical isolates. The amino acid sequence for each gene was concatenated to form a linear consensus sequence for each sample. The amino acid sequences were aligned with MAFFT using the automatic strategy. IQ-Tree analysed the multiple sequence alignment using the TEST model to construct an unrooted phylogenetic tree with 1000 bootstrap replicates.[20] Bootstrap support values were interpreted to determine the likelihood of clinical isolates clustering within the same clade using a similarity index of 1000.
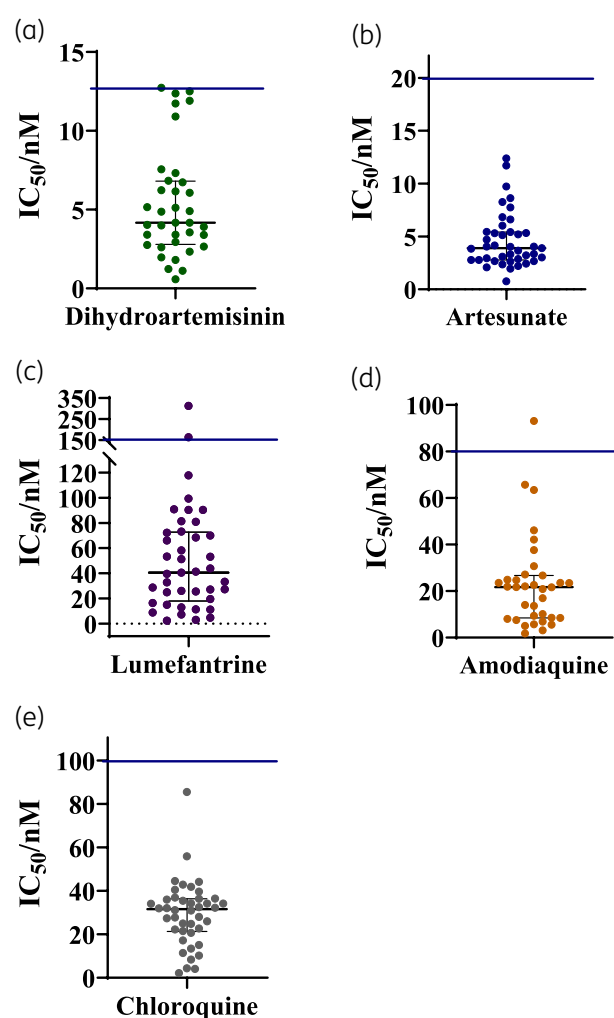
Statistical analysis

IC_{50}s for phenotypic groups were compared using the *Kruskal-Wallis* test. The correlation of lumefantrine, amodiaquine and chloroquine IC_{50} values was determined using Pearson correlation. Distribution of extreme (i.e >median or <median) IC_{50} values of *pfmdr1* Y184F genotypes and pfcrtK76T genotypes was compared using unpaired two-tailed t test. A *P* value of ≤0.05 was considered statistically significant.

Results

Ex vivo *response of freshly isolated* P. falciparum *to five antimalarial drugs*

A total of 66 *ex vivo* growth inhibition assays were performed to test the efficacy of each antimalarial drug against the clinical isolates. High-quality curve fitting and IC_{50} values were obtained for
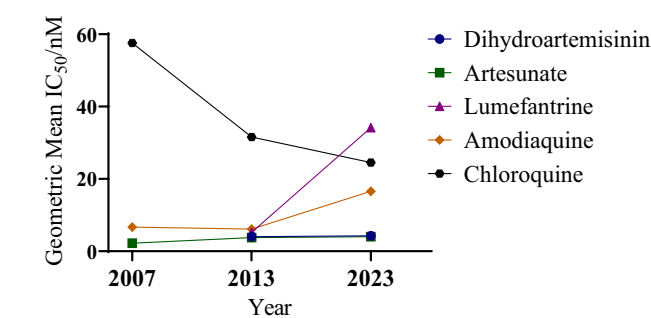

Figure 1. Distribution of *ex vivo* responses of *P. falciparum* clinical isolates to (a) dihydroartemisinin (DHA), (b) artesunate (AS), (c) lumefantrine (LUM), (d) amodiaquine (AQ) and (e) chloroquine (CQ). *P. falciparum* clinical isolates showed variable *ex vivo* responses to DHA, AS, LUM, AQ and CQ. Almost all the clinical isolates tested had IC_{50} values below the resistance cut-offs (indicated by blue line) for DHA, AS, LUM, AQ and CQ.[21–23] Thick black line indicate median with IQR.

48 of the samples (72.7%) across all five drugs. We observed a wide range of *ex vivo* responses to lumefantrine, amodiaquine and chloroquine, with geometric mean IC_{50} values below established resistance thresholds for lumefantrine (150 nM), chloroquine (100 nM) and amodiaquine (60 nM) (Figure 1).[21–23] Amodiaquine had a lower geometric mean IC_{50} value (16.6 nM) compared with lumefantrine (34.2 nM). For chloroquine, a geometric mean IC_{50} of 24.5 nM was observed, which is 4-fold lower than the 100 nM cut-off for chloroquine resistance.[21–23] For dihydroartemisinin, the major active metabolite for artemisinins, a geometric mean IC_{50} value of 4.3 nM was observed, similar to artesunate (4.0 nM), confirming the susceptibility of parasite populations to the artemisinin derivatives in frontline ACTs in Ghana (Table 1). Pearson correlation of IC_{50} values for partner drugs and chloroquine revealed a significant correlation between

Table 1. *Ex vivo* responses of freshly isolated *P. falciparum*

Drug compound	DHA (nM)	AS (nM)	LUM (nM)	AQ (nM)	CQ (nM)
n (assay success, %)	36 (55)	41 (62)	41 (62)	35 (53)	42 (64)
Median	4.17	3.90	40.56	21.71	31.55
IQR	4.01	2.64	54.70	18.27	15.00
Range (min–max)	0.58–12.73	0.76–12.39	2.38–312.40	1.83–93.14	2.11–85.45
Geometric mean	4.30	4.01	34.19	16.56	24.52
(95% CI)	(3.37–5.48)	(3.38–4.78)	(24.51–47.68)	(12.26–22.37)	(19.59–30.68)
3D7 control IC_{50} (nM)	1.65	3.86	54.41	24.11	42.02
Resistance threshold	12	20	150	60	100

DHA, dihydroartemisinin; AS, artesunate; LUM, lumefantrine; AQ, amodiaquine; CQ, chloroquine.

Figure 2. Temporal trends in *ex vivo* responses of *P. falciparum* clinical isolates from 2007, 2013 and 2023.[23,24] There was a minimal increase in the geometric mean IC_{50} values for dihydroartemisinin and artesunate from 2013 to 2023, and 2007 to 2023, respectively (blue and green lines). There was an increase in the geometric mean IC_{50} value for lumefantrine and amodiaquine from 2013 to 2023, and 2007 to 2023, respectively (purple and orange lines). There was a decrease in the geometric mean IC_{50} value for chloroquine from 2007 to 2023 (black line). Artesunate amodiaquine was the only ACT evaluated in the 2007 study, thus no data were present for dihydroartemisinin and lumefantrine.

amodiaquine and chloroquine ($P=0.047$). Lumefantrine and amodiaquine were also correlated ($P=0.045$). There was no significant correlation between amodiaquine and lumefantrine ($P=0.252$) (Figure S3).

Temporal trends in ex vivo *responses of* P. falciparum *clinical isolates*

Temporal changes in the *ex vivo* responses of clinical isolates were evaluated by comparing data from this study with those from similar previous studies conducted in the same settings.[23,24] There was minimal increase in the geometric mean IC_{50} value for dihydroartemisinin and artesunate over time, suggesting their continual efficacy from 2007 to 2023. There was an increase in the geometric mean IC_{50} value of lumefantrine (5.2 nM to 34.2 nM) and amodiaquine (6.6 nM to 16.6 nM) between 2013 and 2023, suggesting selection and reduced susceptibility to both drugs over the years. However, the increase was steeper for lumefantrine compared with amodiaquine. In contrast, there was a consistent sharp decline in geometric mean IC_{50} value for chloroquine (57.6 nM to 24.0 nM) from 2007 to 2023 (Figure 2).

Phylogenetic analysis

To examine possible shared haplotypes among *P. falciparum* clinical isolates, a neighbour-joining tree was constructed from the concatenated sequences of drug resistance marker alleles in the five variable loci (i.e. *pfcrt*, *pfmdr1*, *pfdhfr*, *pfdhps* and *pfkelch13*). This revealed three distinct phylogenetic groups (Figure 3a), mainly driven by *pfdhfr* and *pfdhps* haplotype clusters. The distribution of IC_{50} values for each group by drug did not reveal a clear pattern of susceptibility (Figure 3b–f). However, there was a wide range of responses to each drug within each phylogenetic group. A pairwise comparison of the median IC_{50} values between groups for each drug was generally not statistically significant after Bonferroni correction, except for the artemisinin derivative artesunate (group 1 versus group 2, $P=0.02$ and group 2 versus group 3, $P=0.045$).
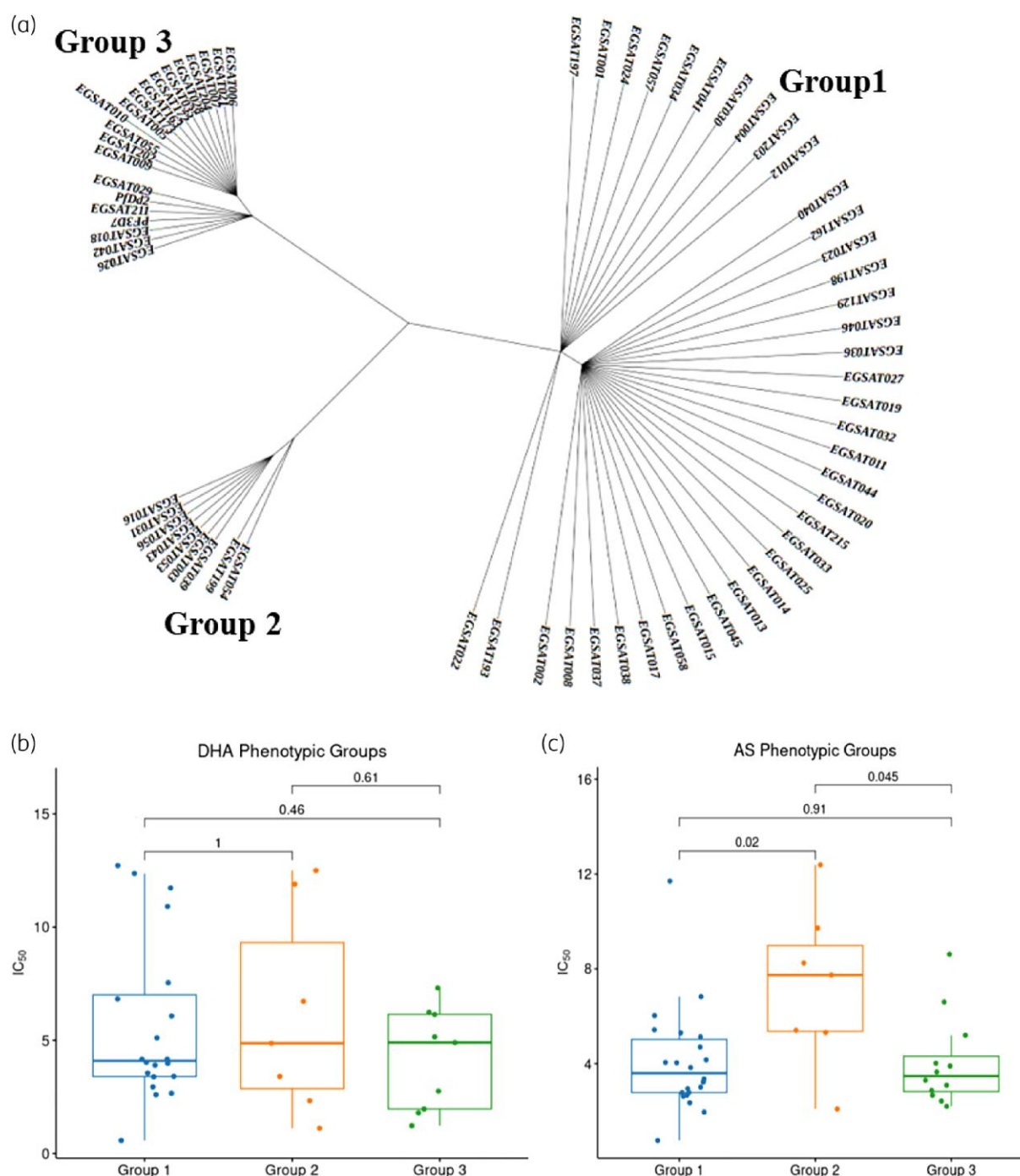
Polymorphism in pfcrt *and* pfmdr1 *and* ex vivo *drug response*

We evaluated the prevalence of polymorphisms in *pfcrt* and *pfmdr1* and parasite drug responses. We analysed samples with above-median IC_{50} values (tolerant isolates) and below-median IC_{50} values (susceptible isolates). For *pfcrt*, only 3% of samples carried the *pfcrt* 76T mutation associated with chloroquine resistance. For *pfmdr1*, 62% of the samples had the *pfmdr1* 184F mutation and there was no mutation at the *pfmdr1* 86 codon (Table S1). There was no significant differences in the *ex vivo* responses (lumefantrine, amodiaquine and chloroquine) of clinical isolates carrying *pfmdr1* Y184 WT or Y184F mutant alleles. However, the majority of the clinical isolates with very high IC_{50} values (outliers) for all drugs tested carried the *pfmdr1* Y184F mutant allele (Figure S4 and Table S2). Except for chloroquine, the *pfmdr1* Y184F mutant was more prevalent in isolates with IC_{50} values greater than the median for each drug tested (Table 2).

Discussion

Antimalarial drug resistance is a major roadblock to global malaria elimination and eradication.[1,25] Following reports of emergence of artemisinin resistance in Cambodia in 2007–08, and its subsequent local spread and/or multiple independent emergence in countries within the Greater Mekong region, significant efforts were made to contain artemisinin-resistant alleles from spreading from SEA to Africa.[3,5,26] However, between 2016 and 2020, validated

Figure 3. Phylogenetic relationship between the phenotyped *P. falciparum* clinical isolates and distribution of IC$_{50}$ values of phylogenetic groups. Phylogenetic analysis of the clinical isolates revealed three distinct phylogenetic groups (a). A comparison of IC$_{50}$s of the three phylogenetic groups revealed no statistically significant difference among the groups for (b) dihydroartemisinin (DHA), (d) lumefantrine (LUM), (e) amodiaquine (AQ) and (f) chloroquine (CQ). There was a statistically significant difference among the phylogenetic groups for (c) artesunate (AS) (group 1 versus group 2, and group 2 versus group 3).

pfkelch13 artemisinin-resistant mutations have independently emerged in the Horn of Africa (in Eritrea and Ethiopia), Uganda, Rwanda and, more recently, Tanzania in 2022.[7–10,27,28] Therefore, the threat of artemisinin resistance in sSA remains high, and continual surveillance is imperative. This study investigated *ex vivo*

responses of *P. falciparum* clinical isolates to frontline antimalarial drugs in Ghana and the contribution of validated drug resistance markers.

Artemisinins remain the most effective antimalarial drugs against *P. falciparum* clinical isolates in the study population.
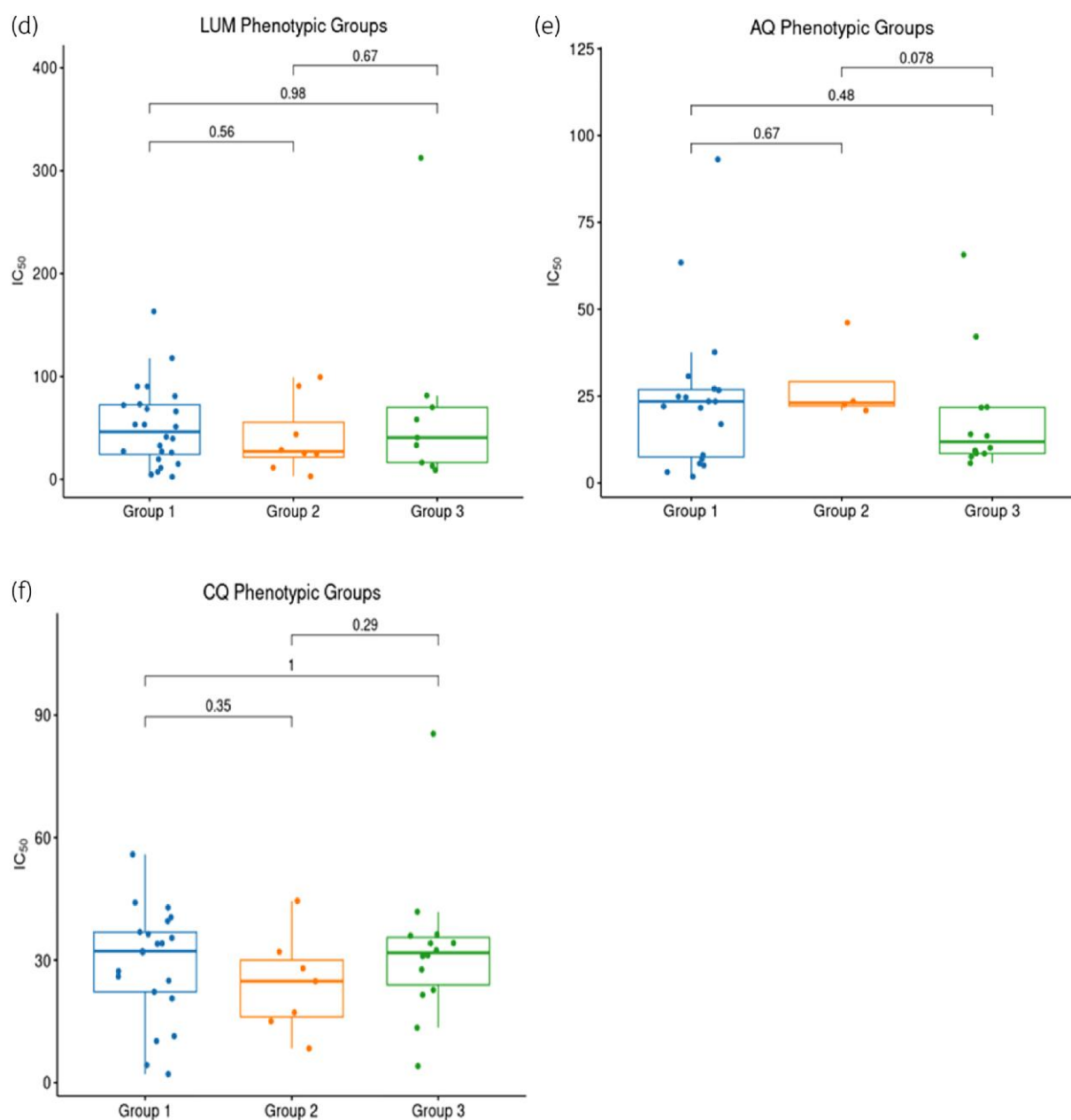
(d)

(e)

(f)

Figure 3. Continued

These findings are reassuring as partial artemisinin resistance is yet to be established in Ghana and most of West Africa. On the contrary, lumefantrine and amodiaquine had relatively higher geometric mean IC_{50} values, suggesting that *P. falciparum* clinical isolates may be gaining some level of tolerance to these commonly used partner drugs in Ghana. Indeed, recent studies in Burkina Faso and Uganda found decreased susceptibility to lumefantrine.[29,30] There are also anecdotal reports from The Gambia.[11,12] Responses to partner drugs had the highest IC_{50} ranges, with substantial variability suggesting incipient adaptation of *P. falciparum* clinical isolates to partner drug pressure ahead of the artemisinin derivatives. Interestingly, in neighbouring Burkina Faso, clinical trials involving artemether/lumefantrine and artesunate/amodiaquine found positive selection of *pfcrt*-K76 and *pfmdr1*-N86 alleles after treatment with artemether/lumefantrine and, conversely, selection of *pfcrt*-76T and *pfmdr1*-86Y alleles after treatment with artesunate/amodiaquine, prompting the suggestion that artemether/lumefantrine and artesunate/amodiaquine impose opposite trends in selecting *pfcrt*-K76T and *pfmdr1*-N86Y alleles.[31,32] This pattern of selection was not observed in the current study, and we believe the use of multiple ACT first-line treatment options in Ghana did not allow the independent selection effects exerted by artemether/lumefantrine

Table 2. Prevalence polymorphisms in *pfcrt* and *pfmdr1*, and *ex vivo* responses[a]

Drug	Comparison (IC$_{50}$ nM)	n	K76T n (%)		Y184F n (%)		K76T/Y184F n (%)		
			WT	M	WT	M	WT/WT	WT/M	M/M
DHA	>median	17	16 (94.1)	1 (5.9)	2 (11.8)	15 (88.2)	2 (11.7)	14 (82.4)	1 (5.9)
	<median	19	18 (94.7)	1 (5.3)	13 (68.4)	6 (31.6)	13 (68.4)	5 (26.3)	1 (5.3)
AS	>median	20	20 (100)	0 (0.0)	7 (35.0)	13 (65.0)	7 (35.0)	13 (65.0)	0 (0.0)
	<median	21	19 (90.5)	2 (9.5)	8 (38.1)	13 (61.9)	8 (38.1)	11 (52.4)	2 (9.5)
LUM	>median	20	19 (95.0)	1 (5.0)	4 (20)	16 (80.0)	4 (20)	15 (75.0)	1 (5.0)
	<median	21	20 (95.2)	1 (4.8)	9 (42.9)	12 (57.1)	8 (38.1)	12 (57.1)	1 (4.8)
AQ	>median	17	16 (94.1)	1 (5.9)	4 (23.5)	13 (76.5)	4 (23.5)	12 (70.6)	1 (5.9)
	<median	18	17 (94.4)	1 (5.6)	8 (44.5)	10 (55.5)	8 (41.5)	9 (52.9)	1 (5.6)
CQ	>median	21	20 (95.2)	1 (4.8)	8 (38.1)	13 (61.9)	8 (38.1)	12 (57.1)	1 (4.8)
	<median	21	21 (100)	0 (0.0)	7 (33.3)	14 (66.7)	7 (33.3)	14 (66.7)	0 (0.0)

DHA, dihydroartemisinin; AS, artesunate; LUM, lumefantrine; AQ, amodiaquine; CQ, chloroquine.
[a]For each drug, number of clinical isolates (*n*) with greater than median (>) and below median IC$_{50}$s (<) was analysed for polymorphisms in *pfcrt*, *pfmdr1* and both. For polymorphisms, the number of isolates with the WT and mutant (M) genotype is shown. Prevalence, in percentage of mutant alleles or haplotypes, is indicated.

and artesunate/amodiaquine to manifest, as observed elsewhere in the subregion and Africa as a whole.

Chloroquine, which has been withdrawn for close to two decades in Ghana following widespread resistance, showed a lower geometric mean IC$_{50}$ value against the clinical isolates compared with the ACT partner drug lumefantrine, which is highly prescribed in the study population.[15,33] Indeed, the IC$_{50}$ values for *P. falciparum* clinical isolates to chloroquine were all within the susceptible range and had the lowest variability compared with other drugs. This reflects a more uniform reversion to chloroquine susceptibility in the Ghanaian *P. falciparum* clinical isolates, possibly driven by the absence of substantial drug selection pressure.

Integration of data from the current study with data from previous similar studies revealed striking temporal trends in parasite response to artemisinin derivatives and the partner drugs lumefantrine and amodiaquine.[23,24] There was marginal increase in the geometric mean IC$_{50}$ value for dihydroartemisinin and artesunate over the three timepoints spanning over 15 years, which shows the continual efficacy of these drugs in Ghana. On the contrary, the temporal data shows high to moderate increases in the geometric mean IC$_{50}$ values for lumefantrine and amodiaquine, respectively, further corroborating possible parasite adaptation to these partner drugs over time. These findings are consistent with another report that showed reduced cure rates for artemether/lumefantrine and artesunate/amodiaquine in Ghana.[34] Taken together, the evidence suggests that ACT partner

drug tolerance may be emerging in Ghana, making continuous molecular surveillance of these drugs imperative. The geometric mean IC$_{50}$ value for chloroquine rapidly decreased over the three timepoints, suggesting gains in chloroquine efficacy, as observed across other malaria-endemic areas where chloroquine has been replaced with ACTs.[35–37]

Targeted amplicon sequencing did not reveal validated *pfkelch13* mutations that are known to confer partial artemisinin resistance, as previously observed in other studies.[38,39] These results are consistent with the *ex vivo* drug response data, where low IC$_{50}$ values were observed for dihydroartemisinin and artesunate. Almost all the clinical isolates tested carried the *pfcrt* K76 WT allele, which strongly supports the expansion of chloroquine-susceptible parasites.[35–37] The three phylogenetic groups obtained did not have any clear pattern of dihydroartemisinin, artesunate, lumefantrine, amodiaquine and chloroquine susceptibility. This suggests that the known drug resistance markers do not form haplotype clusters that correlate with phenotypic resistance in the *P. falciparum* clinical isolates. There are no known validated parasite polymorphisms that have been well characterized to confer tolerance (or resistance) to artemether/lumefantrine and amodiaquine. However, we looked for prevalence of polymorphisms in two key parasite transporter genes, *pfcrt* and *pfmdr1*, and parasites in the tails of IC$_{50}$ distribution (>median versus <median IC$_{50}$) of drug susceptibility. Evaluations of *pfcrt* were not informative as the *pfcrt* K76 WT allele was nearly fixed in the samples tested.

Interestingly, outlier parasites with high IC_{50} values had a greater prevalence of mutant allele at the *pfmdr1* position Y184F than the isolates with high susceptibility. This, together with the fact that, overall, 62.1% isolates carried the mutant *pfmdr1* Y184F allele, suggests that the *Pf*MDR1-N86-184F haplotype, which was significantly over-represented in isolates with high IC_{50} values, may be under selection, in line with reports from other malaria-endemic settings where artemether/lumefantrine is the common first-line treatment for uncomplicated malaria.[40,41] A study in Senegal, where artemether/lumefantrine is largely used, also observed decreased susceptibility to lumefantrine in parasites that carried the N86-Y184F haplotype.[42] Parasites that carried *pfmdr1* N86 WT alleles were associated with an approximately 4.7-fold increase in the odds of artemether/lumefantrine treatment failure.[43] These findings further support accumulating evidence that decreased lumefantrine susceptibility in African *P. falciparum* isolates may be driven by the *pfmdr1* N86 allele alone, or together with other *pfmdr1* alleles and *pfcrt* K76.[41,44,45] Overall, both our *ex vivo* and targeted sequence data reveal patterns of artemisinin and ACT partner drug tolerance, which highlight the need for surveillance of known antimalarial drug resistance markers and for rapid elucidation of mechanisms underlying partner drug resistance to pave the way for molecular marker discovery to support global monitoring of ACTs.

The ring-stage survival assay (RSA), which is recommended for phenotyping artemisinin resistance, was not carried out, thus we recognize the limitation of the growth inhibition assay (IC_{50} values) for determining partial artemisinin resistance.[46,47] Thus, caution needs to be exercised in interpreting dihydroartemisinin and artesunate IC_{50} values. Copy number amplification of *pfmdr1*, which is associated with susceptibility to lumefantrine, also was not determined in the current study but could confound the interpretation of the genotypes at this locus.[48,49]

Conclusions

We have demonstrated sustained susceptibility of clinical isolates in Ghana against artemisinin derivatives (dihydroartemisinin and artesunate), confirmed by the absence of any validated *pfkelch13* mutations. However, the wide distribution of susceptibility to lumefantrine and amodiaquine suggests the possible emergence of ACT partner drug tolerance. The *Pf*MDR1 N86-184F haplotype in combination with *Pf*CRT K76 may be driving this lumefantrine and/or amodiaquine tolerance in this population.

Supplementary data

Figures S1 to S4 and Tables S1 and S2 are available as Supplementary data at *JAC* Online.

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