Age-dependend Selection of Chloroquine-sensitive Plasmodium Falciparum in Some part of Central Region of Ghana

Kwame Kumi Asare (✉ kwamsare@hotmail.com)

University of Cape Coast <https://orcid.org/0000-0002-5219-763X>

Justice Afrifa

University of Cape Coast

Kwaku Opoku Yeboah

University of Education Winneba

Research

Keywords: P. falciparum, chloroquine resistance parasites, chloroquine sensitive parasites, Pfcrt, Pfmdr1, Central Region, Ghana

Posted Date: February 18th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-208111/v1>

License: © ⓘ This work is licensed under a Creative Commons Attribution 4.0 International License.
[Read Full License](#)

Age-depended selection of chloroquine-sensitive *Plasmodium falciparum* in some part of Central Region of Ghana

Kwame Kumi Asare ^{1*}, Justice Afrifa ², Yeboah Kwaku Opoku ³

¹ Department of Biomedical Sciences, School of Allied Health Sciences, College of Health and Allied Sciences, University of Cape Coast, Cape Coast, Ghana

² Department of Medical Laboratory Science, University of Cape Coast, Cape Coast, Ghana

³ Department of Biology Education, Faculty of Science Education, University of Education, Winneba, Ghana

*Corresponding author

Abstract

Introduction

The return of chloroquine-sensitive *P. falciparum* in sub-Saharan Africa countries offers the opportunity for reintroduction of chloroquine either in combination with other drugs or as a single therapy for the management of malaria. The reintroduction of chloroquine can serve as a stopgap to salvage the impending danger of complete failure of malaria treatment due to artemisinin drug resistance. Further, chloroquine reintroduction requires the understanding of the underlying factors that influence the reemergence of chloroquine-sensitive *P. falciparum* in the endemic areas. This study assesses the effects of age on the pattern for selection of CQ sensitive *P. falciparum* markers in the Central Region of Ghana

Methodology

Genomic DNA was extracted from an archived filter paper blood blot from Cape Coast, Elmina, Assin Foso and Twifo Praso using Chelex DNA extraction method. The age information to each extracted sample was collected. The prevalence of chloroquine-sensitive genotyping of Pfcrf K76 and Pfmdr1 N86 was assessed using nested PCR and RFLP.

Results

The overall prevalence of CQ sensitive *P. falciparum* marker (Pfcrf K76) at Central Region of Ghana was 66.36%, whereas the prevalence of Pfcrf K76 at Cape Coast, Assin Foso, Twifo Praso and Elmina were 71.74%, 65.22%, 66.67% and 61.54% respectively. The prevalence of Pfcrf K76 among the age

categories showed that 0-5 years category predominantly selects CQ sensitive Pfprt K76 marker at Cape Coast (34.76%), Assin Foso (37.68%) and Twifo Praso (39.98%). In the case of Pfmdr1 N86, the total prevalence was 84.11% with Cape Coast having 64%, Elmina with 92.26%, Assin Foso with 88.39% and Twifo Praso with 89.91%. There was strong correlation of reemergence of chloroquine-sensitive malaria parasites between Cape Coast and Assin Foso, ($r=0.8568$, $p=0.0318$) Cape Coast and Twifo Praso ($r=0.8671$, $p=0.0285$) and Assin Foso and Twifo Praso, ($r=0.9913$, $p=0.0005$).

Conclusion

The study showed that the selection and expansion of chloroquine-sensitive *P. falciparum* are influenced by age and geographical area. This finding has a significant implication for the future treatment, management and control of *P. falciparum* malaria.

Keywords: *P. falciparum*, chloroquine resistance parasites, chloroquine sensitive parasites, Pfprt, Pfmdr1, Central Region, Ghana

Introduction

The return of chloroquine (CQ) sensitive *Plasmodium falciparum* in sub-Saharan Africa is associated with increased prevalence of wildtype genetic phenotypes [1, 2]. CQ had the most suitable therapeutic properties and excellent efficacy for malaria treatment [3]. Until the emergence of *P. falciparum* resistance to CQ and subsequent withdrawal, CQ was widely used for self-malaria treatment [4]. This hampered the malaria control and treatment leading to CQ resistance and its associated high mortality especially among children in sub-Saharan Africa [5, 6]. The emergence of CQ sensitive *P. falciparum* parasites offers the opportunity to introduce this vetoed drug either in combination therapy or as a single antimalaria drug [7, 8]. However, there is a major concern for the rapid reappearance of CQ resistant parasite in sub-Saharan African countries upon reintroduction [9]. Although the reappearance of CQ sensitive parasites have been observed in all countries, the rate of re-emergence varies from place to place [10]. The central region of Ghana has experienced a slow appearance of CQ sensitive *P. falciparum* compared to other parts of the country [11, 12].

The *P. falciparum* Pfprt K76T and Pfmdr1 N86Y resistant markers are well established to be responsible for chloroquine resistance globally [13]. In Ghana, the prevalence of Pfprt K76T and Pfmdr1 N86Y vary from one place to another [12, 15]. In Ghana, 11.6% and 8.1% of Pfprt K76T and Pfmdr1 N86Y respectively has been reported as the least prevalence of the chloroquine-resistant parasite [16]. This is good news as the most successful deployed antimalarial drug could be used as stopgap to salvage the current challenges involved in the emerging artemisinin resistance [17, 18]. There are also no new antimalarial drugs, and lack of effective and efficient malaria vaccines for the treatment of malaria [19, 20]. Chloroquine in a combination with other effective antimalarial drugs could help in biding time for the search of new effective and efficient antimalarial drugs and vaccines [21].

Artesunate-amodiaquine (ASAQ), artemether-lumefantrine (AL) and dihydroartemisinin-piperaquine (DHAP) serves as a first-line antimalarial drug for uncomplicated malaria in Ghana [22]. However, the emergence of artemisinin resistance in *P. falciparum* in South-East Asia and some parts of Africa has led to treatment failure of ACTs [23, 24]. A current survey from 2007-2016 archived samples from Ghana had shown that K13 mutations responsible for both Asian and African artemisinin resistance were prevalent in the Ghanaian *P. falciparum* parasites [25]. The study also frequently identified N599Y, K607E and V637G non-synonymous mutations in the samples [25]. This gives an indication that the Ghanaian *P. falciparum* parasites have the capacity to develop resistance to artemisinin [25, 26].

It is important to arm ourself against the pending danger that may be associated with a complete failure of artemisinin combination therapy. As such a failure could be catastrophic for malaria morbidity and mortality especially in the sub-Saharan region. Therefore, understanding the factors that influence the selection of chloroquine-sensitive *P. falciparum* would provide us with the necessary tools for early detection and containment of reemergence of chloroquine-resistant strains after the reintroduction of chloroquine for malaria treatment. This study analyzes the effects of age on the pattern for selection of CQ sensitive *P. falciparum* markers in the Central Region of Ghana.

Methodology

Study sites

The study was conducted in four districts in the Central Region of Ghana. The samples were selected from two ecological zones with different malaria endemicity. Assin Foso and Twifo Praso in the forest zone has a high *P. falciparum* malaria prevalence compared to Cape Coast and Elmina in the coastal zone which has lower malaria prevalence in Central Region of Ghana [15].

Sample collection

An archived *Plasmodium falciparum* infected blood blot samples from Cape Coast, Elmina, Twifo Praso and Assin Foso were used from for this study [27]. The air-dried blood spots in zip-locked plastic envelopes containing silica gel stored at -20°C in the Department of Biomedical Sciences, University of Cape Coast were extracted using chelex extraction method. The excel data which contains the sample numbers and patient information at the department were obtained for the analysis.

Genomic DNA extraction

The Chelex-saponin DNA extraction method was used. Briefly, discs punched of about 2.5 mm (2x) were made from dried blood spot then transferred into a 1.5 ml Eppendorf tube. 50 µL of 10% saponin and 1 mL of 1x PBS were added to the blood spots in 1.5 ml tubes. The sample and saponin mixture was vortexed and frozen at 4 °C overnight. The filter was washed 3 x with 1 mL of 1 x PBS, followed with 30 µL of 20% chelex and 70 µL of DNase/RNase water. The sample was incubated at 95 °C for 10 min with an intermittent vortex. The gDNA samples were eluted from the filter by centrifuging at

13,000 rpm for 6 min and the supernatant transferred into a sterile 0.5 mL microfuge tube. The extracted gDNA were stored at -20 °C.

Amplification of Pfcr1 and Pfmdr1 by Nested Polymerase Chain Reaction (PCR)

The genome DNA of *P. falciparum* was amplified using primer pairs (P10-1forw: TTGTCGACCTTAACAGATGGCTCAC / P10-1 rev: AATTTCCCTTTTATTTCCTAAATAAGGA for Pfcr1 and P1-1 forw: TTAAATGTTTACCTGCACAACATAGAAATT / P1-1 rev: CTCCACAATAACTTGCAACAGTTCTTA for mdr1, primary reaction and P10 forw: CTTGTCTTGGTAAATGTGCTC / P10 rev: GAACATAATCATACAAATAAAGT for Pfcr1 and P1 forw: TGTATGTGCTGTATTATCAGGA / P1 rev: CTCTTCTATAATGGACATGGTA for secondary reaction). The primary PCR reaction mixture contained 0.2 µM of the primary primer pair, 5 µl DNA template, 1X PCR buffer, 200 µM dNTPs, 1.5 mM MgCl₂ and 1.25 U of *Taq* DNA Polymerase in a 25 µl mixture. The PCR cycling conditions (95 °C for the 30s, [95 °C for 15 s, 53 °C for 1 min, 68 °C for 1 min], 68 °C for 5 min final extension. The secondary PCR was performed using 0.5 µL of the primary PCR reaction and 133.33 nM each of the second primer pairs. The PCR cycling condition was the same except the annealing temperature which was 60 °C for the second PCR. The 1 µl of the secondary PCR products were run on a 2% agarose gel and visualized using a CSL gel documentation system (CSL, UK).

Restriction fragment length polymorphism (RFLP)

The nested PCR products of Pfcr1 were digested with Apo I whereas that of Pfmdr1 were digested with Apo I and Afl III. The digested products (5 µl) were separated on 2% electrophoresis gel, stained with ethidium bromide and viewed under ultraviolet light. The Pfcr1 PCR product contains single Apo I site if the codon 76 of the Pfcr1 gene encodes for lysine (K76). For Pfmdr1 codon 86 was digested with both Apo I for asparagine and Afl III to confirm the tyrosine mutation at position 86 in all the Pfmdr1 samples that were not digested by Apo I. The digested samples were run on 2% agarose gel and visualized using CSL gel documentation system.

Data analysis

The data from RFLP analysis, patient ages and sample collection sites were organized using Microsoft Office Excel 2010 (Microsoft Corporation) and the statistical analyses were performed with GraphPad Prism software, version 8.4.3 (GraphPad Software). All mixed infection of wild type and resistant strains of Pfcr1 K76T and Pfmdr1 N86Y were excluded from the analysis. The ages of the patients were categorized into 0-5 years, 6-15 years, 16-30 years, 31-45 years and above 45 years before using it for further analysis. The data was translated into the prevalence and statistical significance were determined using paired t-test statistics.

Results

The overall prevalence of CQ sensitive *P. falciparum* markers Pfcr1 K76 and Pfmdr1 N86 at Central Region of Ghana were 66.36% and 84.11% respectively. The predominance of Pfcr1 K76 and Pfmdr1 N86 from *P. falciparum* parasites varies slightly among the study sites (Table 1).

The pattern of selection for chloroquine-sensitive markers Pfcr1 K76 gene among *P. falciparum* isolates showed age dependent selections at each of the study sites. The prevalence of Pfcr1 K76 among 0-5 years, 6-15 years, 16-30 years, 31-45 years and greater than 45 years of age were 34.76%, 15.21%, 23.94%, 19.55% and 6.53% respectively for Cape Coast. A similar pattern was observed for Assin Foso and Twifo Praso except at Elmina which showed a different pattern in the Pfcr1 K76 prevalence among the age categories with 5.14%, 7.69%, 43.58%, 23.07% and 20.52% for 0-5 years, 6-15 years, 16-30 years, 31-45 years and greater than 45 years respectively (Figure 1A and 1B).

The Pfcr1 K76 prevalence was compared between study sites. The results showed a significant difference in Pfcr1 K76 prevalence among the age categories between Cape Coast and Assin Foso, $p<0.05$; Cape Coast and Twifo Praso, $p<0.01$ and Assin Foso and Twifo Praso, $p<0.0001$ (Figure 2A). The correlation analysis between the mean difference for the selection of wildtype Pfcr1 K76 gene showed a significant positive association among Cape Coast vs. Assin Foso ($r=0.8568$, $p=0.0318$), Cape Coast vs. Twifo Praso ($r=0.8671$, $p=0.0285$) and Assin Foso vs. Twifo Praso ($r=0.9913$, $p=0.0005$) (Table 2). The individual mean prevalence of Pfcr1 K76 among the age categories showed that the pattern of distribution varies among the study sites with Cape Coast and Assin Foso having minimum and maximum mean distribution respectively. The mean $\pm$ S.E of the Pfcr1 K76 at Cape Coast (20 ± 4.68 , $p=0.013$) and Elmina (23.24 ± 6.452 , $p=0.043$) showed a significant difference in the mean distributions of Pfcr1 K76 among the age categories (Figure 2B, Table 3).

The Pfmdr1 N86Y mutation is well characterized to augment the Pfcr1 K76T mutation to confer high chloroquine resistance. The emergence of chloroquine-sensitive *P. falciparum* is associated with the return of Pfcr1 K76 and Pfmdr1 N86. The selection of Pfcr1 K76 was therefore confirmed with the prevalence of Pfmdr1 among the age categories at the individual study sites. The Pfmdr1 N86 showed a similar pattern of selection of Pfcr1 K76. The prevalence of Pfmdr1 N86 were 34.78%, 10.59%, 23.91%, 19.57% and 6.52% for 0-5 years, 6-15 years, 16-30 years, 31-45 years and >45 years respectively at Cape Coast. Cape Coast, Assin Foso and Twifo Praso showed an identical pattern of Pfmdr1 N86 distribution except for Elmina which exhibited a different pattern of Pfmdr1 N86 distribution among the age categories (Figure 3A).

The distribution showed a difference in the means at the individual levels. The mean $\pm$ S.E of Pfmdr1 N86 among the age categories at individual study sites showed significant difference at Cape Coast (13.92 ± 3.259 , $p=0.0129$) and Elmina (12.30 ± 4.25 , $p=0.0433$) (Figure 3B, Table 3).

The restoration of chloroquine-sensitive gene Pfmdr1 N86 among the study sites showed a positive correlation for Cape Coast vs. Assin Foso ($r=0.8561$, $p=0.0321$), Cape Coast vs. Twifo Praso ($r=0.8664$, $p=0.0287$) and Assin Foso vs. Twifo Praso ($r=0.9914$, $p=0.0005$) (Table 2). The results indicate a strong influence between study sites on the restoration of chloroquine-sensitive *P. falciparum* parasites in the Central Region of Ghana.

Discussion

1
2
3 The recovery of the CQ sensitive *P. falciparum* has come at a time when the treatment and
4 management of malaria are constrained with development of resistance to almost all the most effective
5 antimalarial drugs [28, 29]. Although the current advances and knowledge on malaria biology have
6 the potential to identify drug targets for the development of safe, effective and efficient antimalarial
7 drugs and vaccines, they are yet to produce an effective means to tackle the challenges facing the
8 malaria treatment and management [30, 31]. As exploration for the new treatment strategies,
9 identification of novel drug targets and the design of new antimalarial compounds are on-going, it is
10 important to understand the dynamism and factors that influence the reemergence of CQ sensitive *P.*
11 *falciparum* in the malaria-endemic areas [32, 33]. This could help in the proper way for the
12 reintroduction of CQ as a temporal strategy to control malaria as we wait for the new effective and
13 safe antimalarial drugs and vaccines [34]. This study focuses on the dynamic effects of age and how
14 it influences the selection of CQ sensitive *P. falciparum* in selected areas in the Central Region of
15 Ghana.
16

17
18 The study showed that CQ resistance markers have reduced to a significant level compared to the
19 previous report from the region [35]. Although the steady decline for Pfcr1 T76 and Pfmdr1 Y86 has
20 been very slow especially at regional capital (Cape Coast) of Central Region of Ghana yet the
21 expansion of the CQ sensitive *P. falciparum* strains have increased appreciably [12, 15, 27]. Similar
22 findings on the reappearance of CQ sensitive parasites have been reported in Malawi, Cameroon,
23 Ethiopia, Nigeria, Tanzania and Mozambique [9, 10]. The decrease in CQ resistant markers and
24 subsequent parasite sensitivity to CQ treatment have been demonstrated clinically [36]. The slow
25 appearance of Pfcr1 K76 and Pfmdr1 N86 is an indication that the maintenance of the integrity of CQ
26 resistant phenotypes have fitness cost in the absent of CQ drug pressure [37, 38]. Hence, the reversion
27 of mutant alleles to the wildtype alleles that are sensitive to CQ treatment [38]. Interestingly, a study
28 has reported that in *Anopheles arabiensis* vectors, there is a selective picking and infection with wild-
29 type *P. falciparum* harbouring Pfcr1 K76 and Pfmdr1 N86 genes in human population which harbours
30 Pfcr1 T76 close to 90% among malaria parasites isolates [39, 40].
31

32
33 The host-parasite relationship is essential for the selection of traits which determines the fitness of
34 parasites [41]. The factors such as virulence, resistance, the complexity of the parasite life-cycle and
35 the demographic characteristics of the host influence the parasite selection, adaptation and survival
36 [42, 43]. The study showed that the selection of Pfcr1 K76 and Pfmdr1 N86 alleles among *P.*
37 *falciparum* isolates in Central region of Ghana is predominantly found in the age category 0-5 years
38 and 16-30 years at Cape Coast, Assin Foso and Twifo Praso except in Elmina where the selection was
39 low among 0-5 years old. The result takes a similar pattern of higher *P. falciparum* infection incidence
40 among children compared to adults [44]. A higher infection prevalence among age < 16 years is
41 associated with the age-dependent acquisition of malaria immunity [45]. Thus, the higher selection of
42 CQ sensitive parasites with Pfcr1 K76 and Pfmdr1 N86 phenotypes by age 0-5 years is important for
43 the parasites to avoid immune clearance and enhance propagation. Again, it has been shown that adults
44 with age > 35 years harbour significantly lower proportion of gametocytes carriage compared to
45 children with age < 17 years while 18-30 years form intermediate gametocyte carriage [46]. A higher
46 gametocyte carriage in young children is an indication of transmission dynamics. The result of this
47 study provides a link between the higher gametocyte carriage among young children and selective
48 infection of CQ sensitive parasites in *Anopheles* mosquito vectors within an area endemic with CQ
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

resistant parasites [44-46]. This link explains why reemergence and expansion CQ sensitive parasites are high among 0-5 years after CQ withdrawal.

Also, the recovery of CQ sensitive parasites is determined by eco-epidemiological factors such as migration and vector competence within an area. Thus, the expansion of Pfcr1 K76 and Pfmdr1 N86 observed among the study sites could be as a result of migration. Cape Coast showed a positive correlation between Assin Foso and Twifo Praso for selection CQ sensitive *P. falciparum* whereas Assin Foso showed a strong positive influence on Twifo Praso. A previous report indicated the possibility of Pfcr1 sensitive parasite either upsurged from low levels in the indigenous parasite populations or migrated from bordering countries with persistent CQ drug pressure [47]. This observation supports the idea of migration of CQ sensitive phenotype from a place with persistent CQ pressure [1]. Such migration of Pfcr1 K76 and Pfmdr1 N86 alleles could be due to rapid movements of goods and service as well as human traffics among Cape Coast (the regional capital), Assin Foso (Assin north municipal capital) and Twifo Praso (the district capital of Twifo Heman Lower Denkyira) in a cyclical manner. It has also been shown that transiting from malaria-endemic area to hypoendemic endemic areas exposes people to the changing risks of malaria infections [48, 49]. Thus, the pattern of malaria infection within a community is significantly influenced by the movement of people [49]. The positive correlations for the prevalence of Pfcr1 K76 and Pfmdr1 N86 among three of the study areas take a similar pattern of the spread of drug resistance lineage parasites [50].

However, Elmina showed a different pattern for the selection of chloroquine-sensitive parasites among the age categories compared to the other study sites. Even though Elmina has close proximity to Cape Coast, there is geographical variation in the distribution CQ sensitive genotype between the two areas. A study conducted at the border of China and Myanmar reported divergent in drug resistance genes and in vitro drug sensitivity [51]. This observation could be explained by factors including the behaviours, the used ITNs and protection of young children in Elmina.

In conclusion, the study showed for the first time that the selection and expansion of CQ sensitive parasite are influenced by age and geographical area. It also establishes a link between two previous studies that reported selective infection of wildtype CQ parasite in *Anopheles* vector and high gametocytes carriage among young children. These findings have significant implication for the future treatment, management and control of *P. falciparum* malaria.

Declarations

Ethical clearance and consent to participate

Not applicable

Consent for publication

Not applicable

1
2 ***Availability of data and materials***
3

4
5 Not applicable
6
7

8
9
10 **Funding**

11
12 No funding agent
13
14
15

16
17 **Competing interests**

18
19 None declared
20
21
22

23
24 **Human and Animal Rights**

25
26 Not applicable
27
28

29 **Author contributions statement**

30
31 All authors contributed equally
32
33
34
35

36 **Acknowledgements**
37
38
39
40

41 I am grateful to the Department of Biomedical Sciences, University of Cape Coast laboratory and the
42 medical staffs of St. Francis Xavier hospital, and Twifo Praso district hospital for their technical
43 support.
44
45
46
47
48
49
50
51

52 **Reference**

- 53
54 1. Dhingra SK, Gabryszewski SJ, Small-Saunders JL, Yeo T, Henrich PP, Mok S, Fidock DA.
55 Global spread of mutant PfCRT and its pleiotropic impact on Plasmodium falciparum multidrug
56 resistance and fitness. MBio. 2019 Apr 30;10(2).
57
58 2. Lu F, Zhang M, Culleton RL, Xu S, Tang J, Zhou H, Zhu G, Gu Y, Zhang C, Liu Y, Wang W.
59 Return of chloroquine sensitivity to Africa? Surveillance of African Plasmodium falciparum
60
61
62
63
64
65

- chloroquine resistance through malaria imported to China. *Parasites & vectors*. 2017 Dec 1;10(1):355.
3. Rainsford KD, Parke AL, Clifford-Rashotte M, Kean WF. Therapy and pharmacological properties of hydroxychloroquine and chloroquine in treatment of systemic lupus erythematosus, rheumatoid arthritis and related diseases. *Inflammopharmacology*. 2015 Oct 1;23(5):231-69.
 4. Pfeiffer K, Some F, Müller O, Sie A, Kouyate B, Haefeli WE, Zoungrana A, Gustafsson LL, Tomson G, Sauerborn R. Clinical diagnosis of malaria and the risk of chloroquine self-medication in rural health centres in Burkina Faso. *Tropical Medicine & International Health*. 2008 Mar;13(3):418-26.
 5. Schumacher RF, Spinelli E. Malaria in children. *Mediterranean journal of hematology and infectious diseases*. 2012;4(1).
 6. Petersen I, Eastman R, Lanzer M. Drug-resistant malaria: molecular mechanisms and implications for public health. *FEBS letters*. 2011 Jun 6;585(11):1551-62.
 7. Mekonnen SK, Aseffa A, Berhe N, Teklehaymanot T, Clouse RM, Gebru T, Medhin G, Velavan TP. Return of chloroquine-sensitive *Plasmodium falciparum* parasites and emergence of chloroquine-resistant *Plasmodium vivax* in Ethiopia. *Malaria journal*. 2014 Dec 1;13(1):244.
 8. Lucchi NW, Komino F, Okoth SA, Goldman I, Onyona P, Wiegand RE, Juma E, Shi YP, Barnwell JW, Udhayakumar V, Kariuki S. In vitro and molecular surveillance for antimalarial drug resistance in *Plasmodium falciparum* parasites in Western Kenya reveals sustained artemisinin sensitivity and increased chloroquine sensitivity. *Antimicrobial agents and chemotherapy*. 2015 Dec 1;59(12):7540-7.
 9. Kamugisha E, Bujila I, Lahdo M, Pello-Esso S, Minde M, Kongola G, Naiwumbwe H, Kiwuwa S, Kaddumukasa M, Kironde F, Swedberg G. Large differences in prevalence of Pfcrt and Pfmdr1 mutations between Mwanza, Tanzania and Iganga, Uganda—a reflection of differences in policies regarding withdrawal of chloroquine?. *Acta tropica*. 2012 Feb 1;121(2):148-51.
 10. Dagnogo O, Ako AB, Ouattara L, Dago ND, Coulibaly DN, Touré AO, Djaman JA. Towards a re-emergence of chloroquine sensitivity in Côte d'Ivoire?. *Malaria journal*. 2018 Dec;17(1):1-7.
 11. Asare KK, Boampong JN, Afoakwah R, Ameyaw EO, Sehgal R, Quashie NB. Use of proscribed chloroquine is associated with an increased risk of pfcrt T76 mutation in some parts of Ghana. *Malaria Journal*. 2014 Dec 1;13(1):246.
 12. Mensah BA, Aydemir O, Myers-Hansen JL, Opoku M, Hathaway NJ, Marsh PW, Anto F, Bailey J, Abuaku B, Ghansah A. Antimalarial Drug Resistance Profiling of *Plasmodium falciparum* Infections in Ghana Using Molecular Inversion Probes and Next-Generation Sequencing. *Antimicrobial Agents and Chemotherapy*. 2020 Mar 24;64(4).
 13. Kayode AT, Akano K, Ajogbasile FV, Uwanibe JN, Oluniyi PE, Bankole BE, Eromon PJ, Sowunmi A, Folarin OA, Volkman SK, McInnis B. Polymorphisms in *Plasmodium falciparum* chloroquine resistance transporter (Pfcrt) and multidrug-resistant gene 1 (Pfmdr-1) in Nigerian children 10 years post-adoption of artemisinin-based combination treatments. *International Journal for Parasitology*. 2020 Dec 24.
 14. Abugri J, Ansah F, Asante KP, Opoku CN, Amenga-Etego LA, Awandare GA. Prevalence of chloroquine and antifolate drug resistance alleles in *Plasmodium falciparum* clinical isolates from three areas in Ghana. *AAS Open Research*. 2018;1.

15. ASARE KK. Evaluation of plasmodium falciparum chloroquine resistant markers in selected health facilities in central region after seven years of banning chloroquine treatment in Ghana.
16. Aninagyei E, Duedu KO, Rufai T, Tetteh CD, Chandi MG, Ampomah P, Acheampong DO. Characterization of putative drug resistant biomarkers in Plasmodium falciparum isolated from Ghanaian blood donors. BMC Infectious Diseases. 2020 Dec;20(1):1-1.
17. Figueiredo P, Benchimol C, Lopes D, Bernardino L, do Rosário VE, Varandas L, Nogueira F. Prevalence of pfmdr1, pfcr1, pfdhfr and pfdhps mutations associated with drug resistance, in Luanda, Angola. Malaria Journal. 2008 Dec;7(1):1-7.
18. Cerqueira GC, Cheeseman IH, Schaffner SF, Nair S, McDew-White M, Phyto AP, Ashley EA, Melnikov A, Rogov P, Birren BW, Nosten F. Longitudinal genomic surveillance of Plasmodium falciparum malaria parasites reveals complex genomic architecture of emerging artemisinin resistance. Genome biology. 2017 Dec 1;18(1):78.
19. Winzeler EA, Manary MJ. Drug resistance genomics of the antimalarial drug artemisinin. Genome biology. 2014 Nov 1;15(11):544.
20. Arama C, Troye-Blomberg M. The path of malaria vaccine development: challenges and perspectives. Journal of internal medicine. 2014 May;275(5):456-66.
21. Parhizgar AR, Tahghighi A. Introducing new antimalarial analogues of chloroquine and amodiaquine: a narrative review. Iranian journal of medical sciences. 2017 Mar;42(2):115.
22. Freeman A, Kwarteng A, Febir LG, Amenga-Etego S, Owusu-Agyei S, Asante KP. Two years post affordable medicines facility for malaria program: availability and prices of anti-malarial drugs in central Ghana. Journal of pharmaceutical policy and practice. 2017 Dec 1;10(1):15.
23. World Health Organization. Artemisinin and artemisinin-based combination therapy resistance: status report. World Health Organization; 2016.
24. Haldar K, Bhattacharjee S, Safeukui I. Drug resistance in Plasmodium. Nature Reviews Microbiology. 2018 Mar;16(3):156.
25. Matrevi SA, Opoku-Agyeman P, Quashie NB, Bruku S, Abuaku B, Koram KA, Fox A, Letizia A, Duah-Quashie NO. Plasmodium falciparum kelch propeller polymorphisms in clinical isolates from Ghana from 2007 to 2016. Antimicrobial agents and chemotherapy. 2019 Nov 1;63(11):e00802-19.
26. Duah NO, Matrevi SA, Quashie NB, Abuaku B, Koram KA. Genetic diversity of Plasmodium falciparum isolates from uncomplicated malaria cases in Ghana over a decade. Parasites & vectors. 2016 Dec 1;9(1):416.
27. Asare KK, Boampong JN, Duah NO, Afoakwah R, Sehgal R, Quashie NB. Synergism between Pfcr1 and Pfmdr1 genes could account for the slow recovery of chloroquine sensitive Plasmodium falciparum strains in Ghana after chloroquine withdrawal. Journal of infection and public health. 2017 Jan 1;10(1):110-9.
28. Tyagi RK, Gleeson PJ, Arnold L, Tahar R, Prieur E, Decosterd L, Pérignon JL, Olliaro P, Druilhe P. High-level artemisinin-resistance with quinine co-resistance emerges in P. falciparum malaria under in vivo artesunate pressure. BMC medicine. 2018 Dec;16(1):1-9.
29. Vydyam P, Dutta D, Sutram N, Bhattacharyya S, Bhattacharyya MK. A small-molecule inhibitor of the DNA recombinase Rad51 from Plasmodium falciparum synergizes with the antimalarial drugs artemisinin and chloroquine. Journal of Biological Chemistry. 2019 May 17;294(20):8171-

83.

30. Yadav BS, Chaturvedi N, Marina N. Recent Advances in System Based Study for Anti-Malarial Drug Development Process. *Current Pharmaceutical Design*. 2019 Sep 1;25(31):3367-77.
31. Kumar S, Bhardwaj TR, Prasad DN, Singh RK. Drug targets for resistant malaria: historic to future perspectives. *Biomedicine & Pharmacotherapy*. 2018 Aug 1;104:8-27.
32. Rout S, Patra NP, Mahapatra RK. An in silico strategy for identification of novel drug targets against *Plasmodium falciparum*. *Parasitology research*. 2017 Sep 1;116(9):2539-59.
33. Crowther GJ, Napuli AJ, Gilligan JH, Gagaring K, Borboa R, Francek C, Chen Z, Dagostino EF, Stockmyer JB, Wang Y, Rodenbough PP. Identification of inhibitors for putative malaria drug targets among novel antimalarial compounds. *Molecular and biochemical parasitology*. 2011 Jan 1;175(1):21-9.
34. Hemming-Schroeder E, Umukoro E, Lo E, Fung B, Tomás-Domingo P, Zhou G, Zhong D, Dixit A, Atieli H, Githeko A, Vardo-Zalik A. Impacts of antimalarial drugs on *Plasmodium falciparum* drug resistance markers, Western Kenya, 2003–2015. *The American journal of tropical medicine and hygiene*. 2018 Mar 7;98(3):692-9.
35. Duah NO, Mavei SA, De Souza DK, Binnah DD, Tamakloe MM, Opoku VS, Onwona CO, Narh CA, Quashie NB, Abuaku B, Duplessis C. Increased *pfmdr1* gene copy number and the decline in *pfprt* and *pfmdr1* resistance alleles in Ghanaian *Plasmodium falciparum* isolates after the change of anti-malarial drug treatment policy. *Malaria journal*. 2013 Dec 1;12(1):377.
36. Nsoby SL, Kiggundu M, Nanyunja S, Joloba M, Greenhouse B, Rosenthal PJ. In vitro sensitivities of *Plasmodium falciparum* to different antimalarial drugs in Uganda. *Antimicrobial agents and chemotherapy*. 2010 Mar 1;54(3):1200-6.
37. Gabryszewski SJ, Dhingra SK, Combrinck JM, Lewis IA, Callaghan PS, Hassett MR, Siriwardana A, Henrich PP, Lee AH, Gnädig NF, Musset L. Evolution of fitness cost-neutral mutant *PfCRT* conferring *P. falciparum* 4-aminoquinoline drug resistance is accompanied by altered parasite metabolism and digestive vacuole physiology. *PLoS pathogens*. 2016 Nov 10;12(11):e1005976.
38. Pelleau S, Moss EL, Dhingra SK, Volney B, Casteras J, Gabryszewski SJ, Volkman SK, Wirth DF, Legrand E, Fidock DA, Neafsey DE. Adaptive evolution of malaria parasites in French Guiana: reversal of chloroquine resistance by acquisition of a mutation in *pfprt*. *Proceedings of the National Academy of Sciences*. 2015 Sep 15;112(37):11672-7.
39. Musyoka KB, Kiiru JN, Aluvaala E, Omondi P, Chege WK, Judah T, Kiboi D, Nganga JK, Kimani FT. Prevalence of mutations in *Plasmodium falciparum* genes associated with resistance to different antimalarial drugs in Nyando, Kisumu County in Kenya. *Infection, Genetics and Evolution*. 2020 Mar 1;78:104121.
40. Mharakurwa S, Sialumano M, Liu K, Scott A, Thuma P. Selection for chloroquine-sensitive *Plasmodium falciparum* by wild *Anopheles arabiensis* in Southern Zambia. *Malaria journal*. 2013 Dec;12(1):1-6.
41. Barrett LG, Thrall PH, Burdon JJ, Linde CC. Life history determines genetic structure and evolutionary potential of host–parasite interactions. *Trends in ecology & evolution*. 2008 Dec 1;23(12):678-85.
42. Lambrechts L, Fellous S, Koella JC. Coevolutionary interactions between host and parasite

- genotypes. Trends in parasitology. 2006 Jan 1;22(1):12-6.
43. Shwab EK, Saraf P, Zhu XQ, Zhou DH, McFerrin BM, Ajzenberg D, Schares G, Hammond-Aryee K, Van Helden P, Higgins SA, Gerhold RW. Human impact on the diversity and virulence of the ubiquitous zoonotic parasite *Toxoplasma gondii*. Proceedings of the National Academy of Sciences. 2018 Jul 17;115(29):E6956-63.
 44. Ladeia-Andrade S, Ferreira MU, de Carvalho ME, Curado I, Coura JR. Age-dependent acquisition of protective immunity to malaria in riverine populations of the Amazon Basin of Brazil. The American journal of tropical medicine and hygiene. 2009 Mar 1;80(3):452-9.
 45. Stanisic DI, Fowkes FJ, Koinari M, Javati S, Lin E, Kiniboro B, Richards JS, Robinson LJ, Schofield L, Kazura JW, King CL. Acquisition of antibodies against *Plasmodium falciparum* merozoites and malaria immunity in young children and the influence of age, force of infection, and magnitude of response. Infection and immunity. 2015 Feb 1;83(2):646-60.
 46. Adomako-Ankomah Y, Chenoweth MS, Tocker AM, Doumbia S, Konate D, Doumbouya M, Keita AS, Anderson JM, Fairhurst RM, Diakite M, Miura K. Host age and *Plasmodium falciparum* multiclonality are associated with gametocyte prevalence: a 1-year prospective cohort study. Malaria journal. 2017 Dec 1;16(1):473.
 47. Gharbi M, Flegg JA, Hubert V, Kendjo E, Metcalf JE, Bertaux L, Guérin PJ, Le Bras J. Longitudinal study assessing the return of chloroquine susceptibility of *Plasmodium falciparum* in isolates from travellers returning from West and Central Africa, 2000–2011. Malaria journal. 2013 Dec 1;12(1):35.
 48. Prothero RM. Migration and malaria risk. Health, Risk & Society. 2001 Mar 1;3(1):19-38.
 49. Lynch C, Roper C. The transit phase of migration: circulation of malaria and its multidrug-resistant forms in Africa. PLoS Med. 2011 May 31;8(5):e1001040.
 50. Takala-Harrison S, Laufer MK. Antimalarial drug resistance in Africa: key lessons for the future. Annals of the New York Academy of Sciences. 2015 Apr;1342:62.
 51. Zeng W, Bai Y, Wang M, Wang Z, Deng S, Ruan Y, Feng S, Yang Z, Cui L. Significant divergence in sensitivity to antimalarial drugs between neighboring *Plasmodium falciparum* populations along the eastern border of Myanmar. Antimicrobial agents and chemotherapy. 2017 Feb 1;61(2).

Figure legends

Figure 1: Chloroquine sensitive Pfcrt marker K76 selection is influenced by age categories

- A. A box and whiskers plot showing the prevalence of chloroquine resistant markers for Pfcrt K76T and Pfmdr1 N86Y among the age categories at the individual study sites.
- B. Age dependent selection of Pfcrt K76 marker for chloroquine sensitive parasites. The data is presented in part of whole using a donut chart.

Figure 2: Differential selection of Pfcrt K76 chloroquine sensitive marker at the study sites

- A. Comparison of Pfcrt K76 among the study sites. The data is presented with box and whiskers

using minimum and maximum values. The statistical significance was tested using paired t-test statistics. NS=Not significant; * = $p<0.05$; ** = $p<0.01$; *** = $p<0.0001$. The prefix C-, E-, T- and A- stands for Cape Coast, Elmina, Twifo Praso and Assin Foso respectively.

- B. The distribution of Pfcrt K76 per age categories at the individual study sites. The data is presented in mean with standard error of the mean using the interleaved scatter diagram.

Figure 3: Age and site selection of chloroquine sensitive *P. falciparum* with Pfmdr1 N86 genes.

- A. Pfmdr1 N86 gene selection is influence by age categories at individual study sites. The data is presented in part of whole using a donut chart.
- B. Mean distribution of Pfmdr1 N86 among age categories across the study sites. The data is presented in mean with standard error of the mean across the study sites.
- C. The comparison of prevalence of Pfmdr1 N86 between study sites. The data is presented with box and whiskers using minimum and maximum values with individual prevalence. The statistical significance was tested using paired t-test statistics. NS=Not significant; * = $p<0.05$; ** = $p<0.01$; *** = $p<0.0001$. The prefix C-, E-, T- and A- stands for Cape Coast, Elmina, Twifo Praso and Assin Foso respectively.

Table 1: Prevalence of Pfcrt K76T and Pfmdr1 N86Y at the study sites

Table 2: Variation in the selection of Pfcrt K76 and Pfmdr1 N86 among the study sites

Table 3: Differential selection of chloroquine sensitive Pfcrt K76 and Pfmdr1 N86 among the age categories at the individual study sites

Table 1

	Pfcrt 76 (%)			Pfmdr1 86 (%)	
Study sites	K	K/T	T	N	Y
Cape Coast	71.74	13.04	15.22	64	36
Assin Foso	65.22	11.59	23.19	88.39	11.61
Twifo Praso	66.67	13.33	20	89.91	10.09
Elmina	61.53	20.51	12.82	92.26	7.74

Table 2

Table 2

	PfCrt K76			PfMdr1 N86		
Study site	Mean diff ±S.E (95% CI)	r	p	Mean diff ±S.E (95% CI)	r	p
Cape Coast vs Elmina	0.322 ± 1.807 (-4.674 - 5.338)	-0.1183	0.4249	-1.62 ± 5.629 (-17.25 - 14.01)	-0.1208	0.4236
Cape Coast vs Assin Foso	6.082 ± 2.873 (-1.895 - 14.06)	0.8568	0.0318*	-2.00 ± 2.271 (-8.305 - 4.305)	0.8561	0.0321*
Cape Coast vs Twifo Praso	0.904 ± 1.072 (-2.072 - 3.88)	0.8671	0.0285*	-0.960 ± 2.518 (-7.950 - 6.03)	0.8664	0.0287*
Elmina vs Assin Foso	5.76 ± 3.73 (-4.596 - 16.12)	0.1233	0.4217	-0.38 ± 5.649 (-16.06 - 17.48)	0.1226	0.4222
Elmina vs Twifo Praso	-10.94 ± 5.527 (-26.28 - 4.409)	0.09033	0.4426	0.66 ± 6.058 (-16.16 - 17.48)	0.0893	0.443
Assin Foso vs Twifo Praso	-5.178 ± 1.885 (-10.41 - 0.055)	0.9913	0.0005***	1.04 ± 0.734 (-0.998 - 3.078)	0.9914	0.0005***

Table 3

Table 3

	PfCrt K76			PfMdr1 N86		
Study site	Mean ± S.E (95% CI)	t	p	Mean ± S.E (95% CI)	t	p
Cape coast	20.0 ± 4.68 (7.01 – 32.99)	4.274	0.013*	18.00 ± 4.692 (4.871 - 22.97)	4.271	0.0129*
Elmina	23.24 ± 6.452 (0.99 – 39.01)	2.92	0.043*	16.30 ± 4.25 (3.599 - 54.00)	2.918	0.0433*
Assin Foso	24.00 ± 7.619 (-0.049 - 40.05)	2.763	0.051	11.92 ± 4.313 (-0.055 - 23.90)	2.764	0.0507
Twifo Praso	26.822 ± 6.764 (-0.077 - 33.721)	2.721	0.052	16.96 ± 4.745 (-0.213 - 26.13)	2.732	0.0524

Figure 1

A

B

Figure 1

A

Figure 2

Figure 3

Figure 3

Figures

Figure 1

Figure 1

Figure 1

Chloroquine sensitive PfCRT marker K76 selection is influenced by age categories A. A box and whiskers plot showing the prevalence of chloroquine resistant markers for PfCRT K76T and PfMDR1 N86Y among the age categories at the individual study sites. B. Age dependent selection of PfCRT K76 marker for chloroquine sensitive parasites. The data is presented in part of whole using a donut chart.

Figure 2

Figure 2

Figure 2

Differential selection of Pfcr K76 chloroquine sensitive marker at the study sites A. Comparison of Pfcr K76 among the study sites. The data is presented with box and whiskers using minimum and maximum values. The statistical significance was tested using paired t-test statistics. NS=Not significant; * = $p<0.05$; ** = $p<0.01$; *** = $p<0.0001$. The prefix C-, E-, T- and A- stands for Cape Coast, Elmina, Twifo Praso and Assin Foso respectively. B. The distribution of Pfcr K76 per age categories at the individual study sites. The data is presented in mean with standard error of the mean using the interleaved scatter diagram.

Figure 3

Figure 3

Age and site selection of chloroquine sensitive *P. falciparum* with Pfmdr1 N86 genes. A. Pfmdr1 N86 gene selection is influence by age categories at individual study sites. The data is presented in part of whole using a donut chart. B. Mean distribution of Pfmdr1 N86 among age categories across the study sites. The data is presented in mean with standard error of the mean across the study sites. C. The comparison of prevalence of Pfmdr1 N86 between study sites. The data is presented with box and whiskers using minimum and maximum values with individual prevalence. The statistical significance was tested using paired t-test statistics. NS=Not significant; * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.0001$. The prefix C-, E-, T- and A- stands for Cape Coast, Elmina, Twifo Praso and Assin Foso respectively.