PLOS ONE

RESEARCH ARTICLE

Prevalence of *pfk13* and *pfmdr1* polymorphisms in Bounkiling, Southern Senegal

Ambroise Ahouidi[1,2]*, **Rafael Oliveira**[3], **Lis Lobo**[3], **Cyrille Diedhiou**[2], **Souleymane Mboup**[2], **Fatima Nogueira**[3]

1 Laboratory of Bacteriology and Virology, Hospital Aristide Le Dantec, Dakar, Senegal, **2** Institute for Health Research, Epidemiological Surveillance and Training (IRESSEF), Dakar, Senegal, **3** Global Health and Tropical Medicine, GHTM, Instituto de Higiene e Medicina Tropical, IHMT, Universidade NOVA de Lisboa, UNL, Lisboa, Portugal

* ambroise.ahouidi@iressef.org

Abstract

Background

Delayed *Plasmodium falciparum* parasite clearance has been associated with Single Nucleotide Polymorphisms (SNPs) in the kelch protein propeller domain (coded by *pfk13* gene). SNPs in the *Plasmodium falciparum* multidrug resistance gene 1 (*pfmdr1)* are associated with multi-drug resistance including the combination artemether-lumefantrine. To our knowledge, this is the first work providing information on the prevalence of k13-propeller and *pfmdr1* mutations from Sédhiou, a region in the south of Senegal.

Methods

147 dried blood spots on filter papers were collected from symptomatic patients attending a hospital located in Bounkiling City, Sédhiou Region, Southern Senegal. All samples were collected between 2015–2017 during the malaria transmission season. Specific regions of the gene *pfk13* and *pfmdr1* were analyzed using PCR amplification and Sanger sequencing.

Results

The majority of parasites (92.9%) harboured the *pfk13* wild type sequence and 6 samples harboured synonymous changes. Regarding *pfmdr1*, wild-type alleles represented the majority except at codon 184. Overall, prevalence of 86Y was 11.9%, 184F was 56.3% and 1246Y was 1.5%. The mutant allele 184F decreased from 73.7% in 2015 to 40.7% in 2017. The prevalence of haplotype NFD decreased from 71.4% in 2015 to 20.8% in 2017.

Conclusions

This study provides the first description of *pfk13* and *pfmdr1* genes variations in Bounkiling, a city in the Sédhiou Region of Senegal, contributing to closing the gap of information on anti-malaria drug resistance molecular markers in southern Senegal.

Introduction

Malaria caused by *P. falciparum* remains a public health problem with the majority of cases and deaths occurring in sub-Saharan Africa [1]. In Senegal, incremental interventions have significantly reduced malaria morbidity and mortality rates [2,3]. Nonetheless, in Senegal half a million people in the country suffered from malaria during 2018 [1].

Prevalence of malaria in Senegal varies across regions, with extremely low prevalence in northern (0.1%) and western (0.2%) regions, and higher prevalence (up to 5.9%) in the southern region [2,3]. Following World Health Organization (WHO) recommendations, in 2006, the Senegalese health authorities changed the first line antimalarial drug to fixed dose artemisinin combination therapies (ACTs); artemether+lumefantrine (AL) and artesunate+amodiaquine (ASAQ), as first and second lines of treatment for uncomplicated *P. falciparum* malaria [4]. Artemisinin resistance, as well as resistance to other antimalarial partner drugs are present in 5 countries of the Greater Mekong subregion (in Southeast Asia) [5–7] and although it has not yet been irrefutably documented in Africa [1], some reports have emerged lately [8–10].

Genome-wide analysis of artemisinin resistance in *P. falciparum* has demonstrated that mutations in the propeller domain of the gene encoding the Kelch 13 (K13) protein (*pfk13*) are associated with resistant *in vitro* and *in vivo* phenotypes in Southeast Asia [6,11,12]. Nine non-synonymous mutations have been validated (F446**I**, N458**Y**, M476**I**, Y493**H**, R5397**T**, I543**T**, P553**L**, R561**H** and C580**Y**) in the *Pfk13* gene as molecular markers, alongside with eleven candidate mutations [13]. In sub-Saharan Africa, increasing frequencies of nonsynonymous mutations on the *pfk13* gene have been reported throughout the continent [14–16] including in Senegal [17,18] and the neighbouring The Gambia [19,20], Guinea-Bissau [21] and Mali [22].

The *P. falciparum multidrug resistance transporter 1* (*pfmdr1*) gene encodes an ABC transporter protein located in the digestive vacuole of the parasite [23]. *Pfmdr1* polymorphisms at codons N86Y, Y184F, S1034C, N1042D, and D1246Y have been associated with drug resistance to several antimalarials [24–26]. Drug pressure (*in vivo*) due to ACT partner drugs has resulted in directional selection of *pfmdr1* variants: 86Y, Y184 and 1246Y for amodiaquine (AMQ) and N86, 184F and D1246 for AL [24–27] The same tendency is also observed *in vitro* and *ex vivo* susceptibility assays [28,29]. Interestingly, allelic replacement of *pfmdr1* 86Y was able to increase parasite susceptibility to dihydroartemisinin (DHA) *in vitro* [30].

Ex vivo susceptibilities to ACT partner drugs, lumefantrine (LUM) and AMQ, have been decreasing since 2008 in Senegal and neighboring The Gambia and Mali [19,20,31]. In The Gambia, some isolates have shown declining sensitivity to DHA in in vitro assays [19]. Despite sustained drug pressure (accessible through public and private health facilities) and a high burden of malaria, few studies on *P. falciparum* molecular markers of drug resistance surveillance are available from southern regions of Senegal [32]. Most of the studies of molecular marker surveillance in Senegal have been performed in Dakar and Thiés [17,31,33–41] (Fig 1). Our study provides information on *pfk13* and *pfmdr1* from Bounkiling, a city located in the Sédhiou region, in the South of Senegal (Fig 1).

Sédhiou has year-round malaria transmission that peaks during the rainy season (June through October) with sustained but lower transmission the rest of the year. This region lies between two malaria endemic countries: The Gambia (66 cases/1000 people at risk) and Guinea-Bissau (123 cases/1000 people at risk) [1].

Materials and methods

Sample collection

In this study, we collected 147 samples (blood spots on Whatman filter paper) from *P. falciparum* infected patients with uncomplicated malaria, attending the health center in Bounkiling,
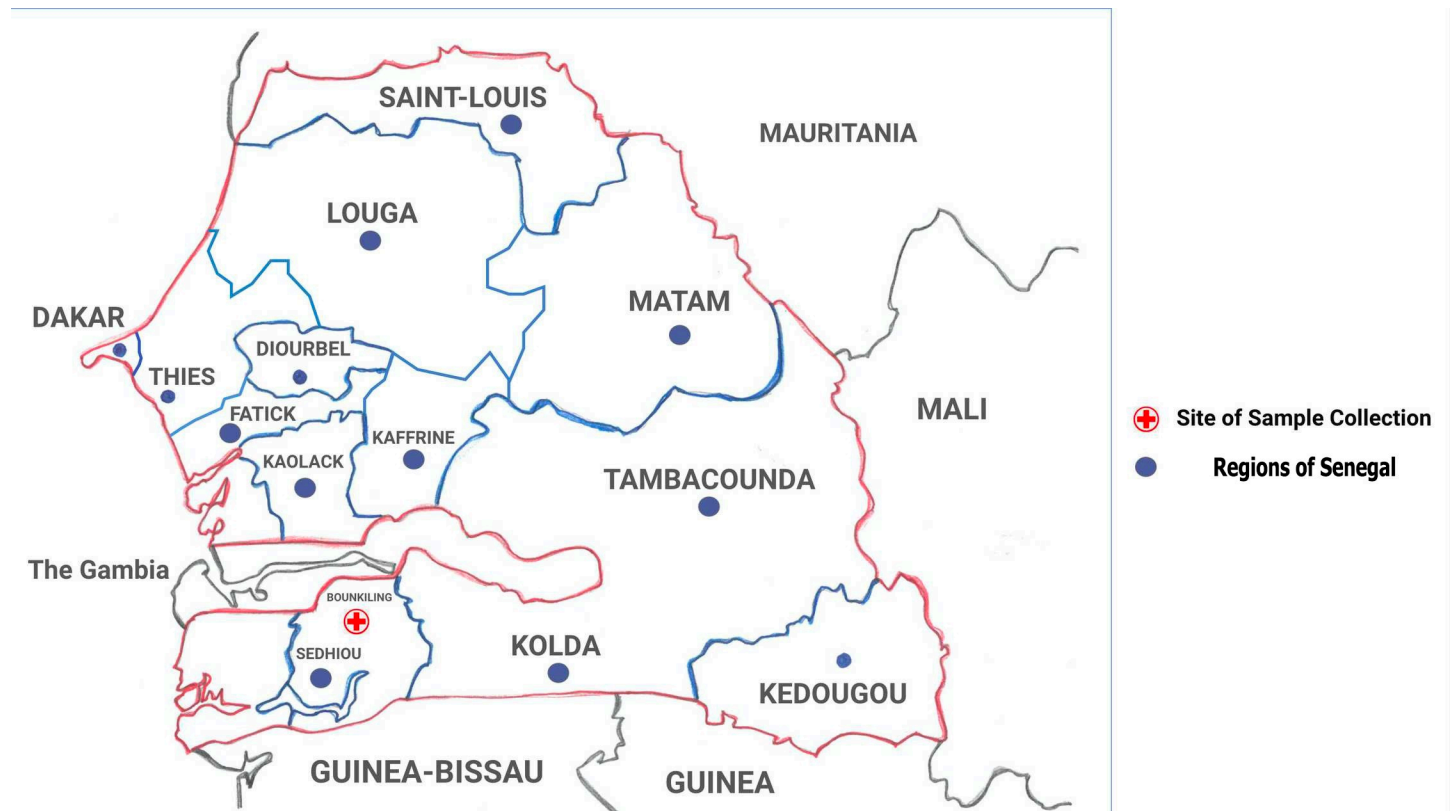
Fig 1. Map of Senegal showing the different regions and the sample collection site.

Senegal. After laboratory confirmation of malaria infection by microscopy or rapid diagnostic test, patients were asked to participate in the study. Written informed consent was obtained from all patients before sample collection. The study was reviewed and approved by the Ethical Committee of the Ministry of Health of Senegal (082 MSAS/DPRS/CNERS). Samples were collected during the peak malaria transmission period (October to December) in 2015, 2016 and 2017.

Genotyping of *pfmdr1* and *pfk13* genes

P. falciparum mutations associated with resistance to ACT components were typed using PCR amplification and Sanger sequencing. We evaluated specific regions of the genes *pfk13* (codon 436 to 706) and *pfmdr1* (codons 86, 184 and 1246). DNA from 147 blood spots was extracted using Chelex method [42] and DNA was stored at -20˚C.

For *pfk13* one fragment containing the main polymorphisms associated with delayed clearance in Southeast Asia was amplified by nested PCR as described elsewhere [43] with modifications. Briefly, specific primers were developed for this purpose (forward—3' GAAAGAAG CAGAATTTTATGG5'; reverse—3' GCTTGGCCCATCTTTATTAGTTCCC 5', obtaining a fragment of 856bp. A semi-nested PCR was performed in some samples using the inner forward primer 3' GTGTAGAATATTTAAATTCG 5' obtaining a fragment of 788bp.

For *pfmdr1*, specific primers were designed for the amplification of the fragment of 452bp containing codons 86 and 184 (forward—3' GTATGTGCTGTATTATCAGGAGGA 5'; reverse—3' TTAATTTATGTTTGTGGTGTCATATG 5') and for the amplification of the fragment of 508bp containing codon 1246 (forward—3' CTACAGCAATCGTTGGAGAA 5'

reverse—3' GAGAATAGCTATAGCTAGAGC 5'). PCR conditions 94˚C 2 min; [94˚C 1 min, 56˚C 1 min, 72˚C 1 min] 10X; [94˚C 1 min, 50˚C 1 min, 72˚C 1 min] 30X; 72˚C 3 min. An aliquot of the PCR products was analyzed by electrophoresis on a 2% agarose gel stained with GreenSafe Premium (Nzytech, Portugal) to confirm single band amplification. All PCR products were then purified using SureClean Plus (Bioline) before Sanger sequencing at Eurofins Genomics (GATC services, Germany).

Data analysis

Sequences were analyzed using Multalin software (http://multalin.toulouse.inra.fr/multalin/multalin.html; free online) using *P. falciparum*-3D7 strain as wild type genotype.

The prevalence of a particular mutant allele was calculated as the proportion of the specific mutant samples among the total number of samples successfully analyzed for this mutation. All graphical representations and calculations were performed with GraphPad Prism PRISM 8 for MAC (V8.4.2).

Results

Sample characteristics

Among the 147 samples analyzed, twenty-one of them tested negative for *P. falciparum* by PCR (6/39 in 2015, 6/65 in 2016 and 9/43 in 2017), hence removed from further analysis. Of the remaining 126 blood samples, 7 had no slide available, so they were not considered for the parasitemia evaluation that varied between 0.02–9.33% with a median value of 0.67%. Of the 126 subjects, 72 (57.14%) were men and 54 (42.86%) were women, and age varied between 3–70 years, with a median of 18 years. The majority 46.83% (n = 59) were adults (over 20 years), 40.48% (n = 51) were adolescents and 12.69% (n = 16) were children between the age of 3 and 10 years.

Molecular markers of anti-malarial drug resistance

All 126 samples (from day 0 before treatment), were successfully sequenced for *pfk13*, 120 were wild type (identical to the reference, 3D7 strain) and 6 carried synonymous mutations (Table 1). One of the synonymous mutations, C469C was previously described in Senegal [17].

Table 1. Mutations in *pfk13* and *pfmdr1* genes.

Year (n)	*pfk13*	*pfmdr1*						
		Allele prevalence (%)						Haplotype (%)
	Codon (*)	N86	86Y	Y184	184F	D1246	1246Y	
2015 (33)	F583F (2)	90.9	9.1	26.3	73.7	100	0	NFD (71.4)
								NYD (14.3)
								YFD (14.3)
2016 (59)	F491F (1)	90.2	9.8	42.0	58.0	100	0	NFD (54.5)
								NYD (40.9)
								YFD (4.5)
2017 (34)	G545G (1)	82.1	17.9	59.3	40.7	96.0	4.0	NFD (20.8)
	C469C (1)							NYD (62.5)
	A627A (1)							YFD (16.7)
overall	6*/126	88.1	11.9	43.8	56.3	98.5	1.5	

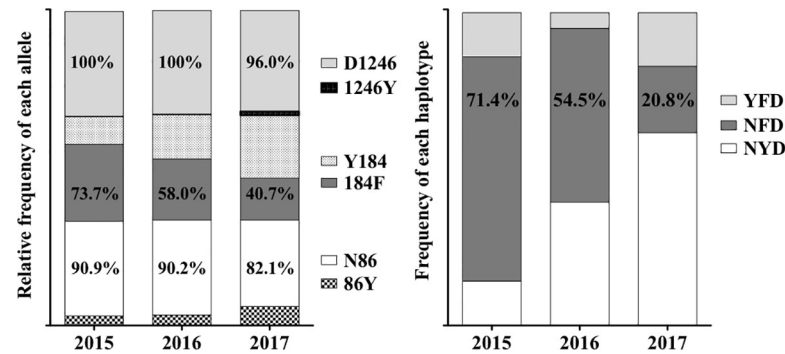*number of samples with synonymous mutation.

Fig 2. Temporal changes of prevalence at codons 86, 184 and 1246 of *pfmdr1*. Percentage (%) inserted in the graphic bars represents the prevalence of the alleles and haplotypes in each year.

https://doi.org/10.1371/journal.pone.0249357.g002

None of the *pfk13* variants associated with artemisinin resistance in Southeast Asia [13] were observed in our study (Table 1).

A total of 101, 96 and 68 samples were successfully genotyped for *pfmdr1* codons 86, 184, and 1246, respectively. Mixed *pfmdr1* alleles were not observed. Overall, wild-type alleles predominated (Fig 2), except for codon 184 where the mutant allele 184F occurred more frequently (56.3%) than the wild-type allele Y184 (43.8%; Table 1). When analyzed separately over time, the prevalence of the mutant allele 184F decreased from 73.7% in 2015 to 58.0% in 2016 and to 40.7% in 2017 (Table 1).

For *pfmdr1*, only three haplotypes were identified with the following overall prevalence: NFD (45.0%), NYD (43.3%) and YFD (11.7%). No triple *pfmdr1* mutants were found at codon 86, 184, or 1246 (YFY). When compared between years, the prevalence of the triple haplotype NFD decreased significantly from 71.4% in 2015 to 54.5% in 2016 and 20.8% in 2017 (Fig 2).

Discussion

Considering the trends of artemisinin decreasing activity [5–7], treatment efficacy should be monitored at regular intervals. Monitoring should be performed by directly measuring efficacy *in vivo*, or indirectly, by molecular markers surveillance, which can reveal drug response trends that inform on the possible therapeutic life-span of a given treatment [13]. In Senegal, ACTs (AL, ASAQ and DHA-piperaquine; DHA-PPQ) remain highly effective for the treatment of uncomplicated *P. falciparum* malaria. In the few reported cases of recrudescence after ACT treatment, parasite samples did not carry mutations in the K13-propeller domain [32]. These observations seem to follow the regional tendency.

In Senegal, there are currently no systematic (nationwide) epidemiological studies on molecular markers of drug resistance. Although a number of studies have been published in Senegal reporting mutations in *pfk13* [20,31,32,36–38,44], samples from those studies originate mostly from Thies and Dakar regions (near and around the capital). In one exception, a recent study reported *pfk13* SNPs from Diourbel (east of Thiés) and Kedougou, in the southeast of the country (Fig 1); [32]. Hence the importance of our study, which analyses samples from Bounkiling City, in the Sédhiou region in the south of Senegal (Fig 1). Our analysis revealed that all but 6 of the isolates carried wild type K13-propeller. This is in line with recent observations in Senegal, where most of the samples were also wild type for K13-propeller domain [17,32,38]. None of the 5 identified SNPs in our samples occurred in more than two samples (Table 1). Nevertheless one study including 207 isolates from Thiés and Dakar [17], identified a total of 22 SNPs in *pfk13* propeller domain (7 synonymous and 15 non-synonymous). In our

work, we have identified only 6 polymorphisms 5 synonymous and one non-synonymous (Table 1). Most of these polymorphisms were limited to single isolates, suggesting that they are likely transient polymorphisms part of naturally evolving parasite populations. The fact that some of these polymorphisms tend to occur in the same positions, might indicate that certain regions of the gene are more disposed to variations then others. Also, *pfk13* artemisinin-resistance associated alleles being selected in Africa, may not be the same as the ones selected in Southeast Asia. The synonymous polymorphism C469C might be particularly relevant because, in this position, two variant amino acids (C469F and C469Y) have been previously associated with slow clearance of parasitemia, after ACT therapy [45,46]. Additionally, this polymorphism was also previously reported in samples from Thiés and Dakar in Senegal [17] and in Ghana [47,48]. In the two countries bordering Sedhiou, The Gambia to the north and Guinea-Bissau to the south (Fig 1), wild type *pfk13* is highly prevalent and SNPs associated with artemisinin resistance in Southeast Asia, have not been identified [19–21]. Nevertheless, monitoring the prevalence of these loci over time may help to infer which alleles are biologically relevant or selected under drug pressure.

The molecular marker *pfmdr1* 86Y (together with *pfcrt*, initially driven through the parasite population by the previous widespread use of chloroquine) has been decreasing in many parts of Africa. This declining prevalence is accelerated in countries using AL, consistent with the expected direction of selection [49,50]. Here we report a high prevalence of wild type alleles N86 and D1246, which follow the tendency reported from northern Senegal, namely from Thiés and Dakar [19,20,28,33,35,44,51–54], the neighbouring The Gambia [19,20,54], and other West Africa countries [16,55]. The *pfmdr1* 86Y is associated with a longer time to reinfection after AL treatment and a shorter time after ASAQ [49], reinforcing its informative value as a molecular marker of antimalarial drug susceptibility.

For codon 184, our analysis of *pfmdr1* revealed differences in the distribution of mutant allele between years. The high prevalence of 184F in 2015 (73.7%) decreased over time to 40.7% in 2017 (Fig 2; Table 1). Declining of 184F prevalence was previously reported in parasite samples collected between 2012 and 2014 in northern Senegal [20,31,51] and in The Gambia [20]. This decrease, in Senegal, was correlated with a local and temporary change to ASAQ as first the line of treatment [31]. Three *pfmdr1* haplotypes were identified in our study: NFD, NYD and YFD. The haplotype YYY was not identified; however, this was not surprising since YYY is believed to be selected under the pressure of DHA-PPQ treatment from a genetic background 86Y and Y184 [56,57]. DHA-PPQ has not been widely used in Senegal, except to compensate for antimalarial drug shortages in 2010 and 2011 [32]. Other studies have reported absence or low prevalence of the allele 1246Y [35] or the haplotype YYY haplotype in Senegal (northern regions) [31]. The two haplotypes NFD and NYD were the most frequent triple haplotypes found in our samples, as was the case in other studies from northern Senegal [31,51] in the neighbouring The Gambia [19,20] and generally in Africa [50]. These observations argue in favor of lumefantrine selecting the triple haplotype N86F184D1246 (NFD) [28,31,35,58–60] and that this selection might be sequential; first N86, second D1246 and the last 184F [24,50,61,62]. A high prevalence of haplotype NFD was observed in 2015 (71.4%) samples, however, a significant decrease was detected in 2017 (20%) driven by the decline of the mutant allele 184F (Fig 2). This decrease could be explained by therapy policy change, and/or intense migration of parasite populations to Bounkiling from other regions of Senegal or the neighboring The Gambia or Guinea-Bissau. Malaria therapy policy did not change in Bounkiling during 2016 and 2017, which could cause decreased in AL pressure hence accounting for the observed decrease in 184F frequency. Recently, a decreasing trend in frequency of the 184F allele around the same period, was reported from the neighboring Guinea-Bissau [21,63]. Hence intense migration of parasite populations to Bounkiling from Guinea-Bissau, could

account (at least in part) for the observed 184F decreasing frequency trend. To the best of our knowledge, there is no published information on the prevalence of these markers between 2015 and 2017 (or since 2017) either from other regions in Senegal or from the neighboring The Gambia. A higher number of samples analyzed with next generation sequencing, would increase the robustness of our results. A national survey of molecular markers of drug resistance would help to clarify these results and better inform National Public Health Authorities on first-line treatment regimens to manage uncomplicated *P. falciparum* malaria.

Conclusions

All isolates carried wild type *pfk13* gene, except for 6 samples that carried 5 different synonymous SNPs. Prevalence of haplotype NFD in pfmdr1 gene, decreased over time from 2015 to 2017. To our knowledge, this is the first work providing information on the prevalence of k13-propeller and *pfmdr1* mutations in Sédhiou, a region in the south of Senegal. We are contributing to the surveillance of molecular markers of drug resistance in Africa.

Supporting information

S1 File. Primers used in this study.
(PDF)

S2 File. Comprehensive data of isolate genotype for *pfmdr1* N86Y, Y184F and D1246Y.
(PDF)

S3 File. Comprehensive data showing sequence alignment for pfK13 with highlighted SNPs.
(PDF)

Acknowledgments

We are grateful to all the patients who participated in the study, to Maria Mendes and Amy Bei for helping with English revision and to the team in Bounliking Dr Jean.S.N. Kaly, Dr Bou Diarra, Elhadj Abou Diop, Ndeye Fatou Thiam Kebe, Mansanou Fall, Theophile Coly and Dior Diop for collecting samples.

Author Contributions

Conceptualization: Ambroise Ahouidi, Fatima Nogueira.

Investigation: Ambroise Ahouidi, Rafael Oliveira, Lis Lobo, Cyrille Diedhiou.

Writing – original draft: Ambroise Ahouidi.

Writing – review & editing: Souleymane Mboup, Fatima Nogueira.

References

1. WHO. World Malaria Report 2019. Geneva. World Malar Rep. 2019.

2. Programme National de Lutte Contre le Paludisme. Bulletin Epidemiologique Annuel 2017 du Paludisme au Senegal. Ministere de la Sante. 2018. p. 1–40.

3. Faye S, Cico A, Gueye AB, Baruwa E, Johns B, Ndiop M, et al. Scaling up malaria intervention "packages" in Senegal: Using cost effectiveness data for improving allocative efficiency and programmatic decision-making. Malar J. 2018. https://doi.org/10.1186/s12936-018-2305-6 PMID: 29636051

4. Programme National de Lutte Contre le Paludisme. Progress & Impact Series: Focus on Senegal. Roll Back Malaria. 2010. p. 1–54.

5. Dondorp AM, Nosten F, Yi P, Das D, Phyo AP, Tarning J, et al. Artemisinin resistance in Plasmodium falciparum malaria. N Engl J Med. 2009. https://doi.org/10.1056/NEJMoa0808859 PMID: 19641202

6. Takala-Harrison S, Jacob CG, Arze C, Cummings MP, Silva JC, Dondorp AM, et al. Independent emergence of artemisinin resistance mutations among Plasmodium falciparum in Southeast Asia. J Infect Dis. 2015. https://doi.org/10.1093/infdis/jiu491 PMID: 25180241

7. Imwong M, Suwannasin K, Kunasol C, Sutawong K, Mayxay M, Rekol H, et al. The spread of artemisinin-resistant Plasmodium falciparum in the Greater Mekong subregion: a molecular epidemiology observational study. Lancet Infect Dis. 2017; 17(5):491–7. https://doi.org/10.1016/S1473-3099(17)30048-8 PMID: 28161569

8. Ebohon O, Irabor F, Ebohon LO, Omoregie ES. Therapeutic failure after regimen with artemether-lumefantrine combination therapy: A report of three cases in Benin city, Nigeria. Rev Soc Bras Med Trop. 2019. https://doi.org/10.1590/0037-8682-0163-2019 PMID: 31618306

9. Van Hong N, Amambua-Ngwa A, Tuan NQ, Cuong DD, Giang NTH, Van Dung N, et al. Severe malaria not responsive to artemisinin derivatives in man returning from Angola to Vietnam. Emerg Infect Dis. 2014.

10. Dahal P, d'Alessandro U, Dorsey G, Guerin PJ, Nsanzabana C, Price RN, et al. Clinical determinants of early parasitological response to ACTs in African patients with uncomplicated falciparum malaria: a literature review and meta-analysis of individual patient data. BMC Med. 2015. https://doi.org/10.1186/s12916-015-0445-x PMID: 26343145

11. Takala-Harrison S, Clark TG, Jacob CG, Cummings MP, Miotto O. Genetic loci associated with delayed clearance of Plasmodium falciparum following artemisinin treatment in Southeast Asia. Proc Natl Acad Sci. 2013; 110(1):240–5. https://doi.org/10.1073/pnas.1211205110 PMID: 23248304

12. Ariey F, Witkowski B, Amaratunga C, Beghain J, Langlois AC, Khim N, et al. A molecular marker of artemisinin-resistant Plasmodium falciparum malaria. Nature. 2014. https://doi.org/10.1038/nature12876 PMID: 24352242

13. WHO. Artemisinin resistance and artemisinin-based combination therapy efficacy: status report. [Internet]. 2018. Available from: https://apps.who.int/iris/handle/10665/274362.

14. Djaman JA, Olefongo D, Ako AB, Roman J, Ngane VF, Basco LK, et al. Molecular epidemiology of malaria in Cameroon and Côte d'Ivoire. XXXI. Kelch 13 propeller sequences in plasmodium falciparum isolates before and after implementation of artemisinin-based combination therapy. Am J Trop Med Hyg. 2017.

15. Dorkenoo AM, Yehadji D, Agbo YM, Layibo Y, Agbeko F, Adjeloh P, et al. Therapeutic efficacy trial of artemisinin-based combination therapy for the treatment of uncomplicated malaria and investigation of mutations in k13 propeller domain in Togo, 2012–2013. Malar J. 2016. https://doi.org/10.1186/s12936-016-1381-8 PMID: 27334876

16. Ocan M, Akena D, Nsobya S, Kamya MR, Senono R, Kinengyere AA, et al. K13-propeller gene polymorphisms in Plasmodium falciparum parasite population in malaria affected countries: A systematic review of prevalence and risk factors. Malaria Journal. 2019.

17. Talundzic E, Ndiaye YD, Deme AB, Olsen C, Patel DS, Biliya S, et al. The molecular epidemiology of Pf k13 mutations in Senegal using targeted amplicon deep sequencing. Antimicrob Agents Chemother. 2017; 61(3):1–8.

18. Boussaroque A, Fall B, Madamet M, Wade KA, Fall M, Nakoulima A, et al. Prevalence of anti-malarial resistance genes in Dakar, Senegal from 2013 to 2014. Malar J. 2016. https://doi.org/10.1186/s12936-016-1379-2 PMID: 27387549

19. Amambua-Ngwa A, Okebe J, Mbye H, Ceesay S, El-Fatouri F, Joof F, et al. Sustained ex vivo susceptibility of Plasmodium falciparum to artemisinin derivatives but increasing tolerance to artemisinin combination therapy partner quinolines in The Gambia. Antimicrob Agents Chemother. 2017. https://doi.org/10.1128/AAC.00759-17 PMID: 28971859

20. Dieye B, Affara M, Sangare L, Joof F, Ndiaye YD, Gomis JF, et al. West Africa international centers of excellence for malaria research: Drug resistance patterns to artemether-lumefantrine in Senegal, Mali, and the Gambia. Am J Trop Med Hyg. 2016.

21. Nag S, Ursing J, Rodrigues A, Crespo M, Krogsgaard C, Lund O, et al. Proof of concept: Used malaria rapid diagnostic tests applied for parallel sequencing for surveillance of molecular markers of anti-malarial resistance in Bissau, Guinea-Bissau during 2014–2017. Malar J. 2019. https://doi.org/10.1186/s12936-019-2894-8 PMID: 31349834

22. Ouattara A, Kone A, Adams M, Fofana B, Maiga AW, Hampton S, et al. Polymorphisms in the K13-propeller gene in artemisinin-susceptible Plasmodium falciparum parasites from Bougoula-Hameau and Bandiagara, Mali. Am J Trop Med Hyg. 2015. https://doi.org/10.4269/ajtmh.14-0605 PMID: 25918205

23. Cowman AF, Karcz S, Galatis D, Culvenor JG. A P-glycoprotein homologue of Plasmodium falciparum is localized on the digestive vacuole. J Cell Biol. 1991; 113(5):1033–42. https://doi.org/10.1083/jcb.113.5.1033 PMID: 1674943

24. Humphreys GS, Merinopoulos I, Ahmed J, Whitty CJM, Mutabingwa TK, Sutherland CJ, et al. Amodiaquine and artemether-lumefantrine select distinct alleles of the Plasmodium falciparum mdr1 gene in Tanzanian children treated for uncomplicated malaria. Antimicrob Agents Chemother. 2007. https://doi.org/10.1128/AAC.00875-06 PMID: 17194834

25. Dokomajilar C, Nsobya SL, Greenhouse B, Rosenthal PJ, Dorsey G. Selection of Plasmodium falciparum pfmdr1 alleles following therapy with artemether-lumefantrine in an area of Uganda where malaria is highly endemic. Antimicrob Agents Chemother. 2006.

26. Baraka V, Tinto H, Valea I, Fitzhenry R, Delgado-Ratto C, Mbonye MK, et al. In vivo selection of plasmodium falciparum pfcrt and pfmdr1 variants by artemether-lumefantrine and dihydroartemisinin-piperaquine in Burkina Faso. Antimicrob Agents Chemother. 2015. https://doi.org/10.1128/AAC.03647-14 PMID: 25403659

27. Price RN, Uhlemann AC, Brockman A, McGready R, Ashley E, Phaipun L, et al. Mefloquine resistance in Plasmodium falciparum and increased pfmdr1 gene copy number. Lancet. 2004. https://doi.org/10.1016/S0140-6736(04)16767-6 PMID: 15288742

28. Wurtz N, Fall B, Pascual A, Fall M, Baret E, Camara C, et al. Role of Pfmdr1 in In Vitro Plasmodium falciparum Susceptibility to Chloroquine, Quinine, Monodesethylamodiaquine, Mefloquine, Lumefantrine, and Dihydroartemisinin. Antimicrob Agents Chemother. 2014.

29. Dama S, Niangaly H, Ouattara A, Sagara I, Sissoko S, Traore OB, et al. Reduced ex vivo susceptibility of Plasmodium falciparum after oral artemether-lumefantrine treatment in Mali. Malar J. 2017. https://doi.org/10.1186/s12936-017-1700-8 PMID: 28148267

30. Veiga MI, Dhingra SK, Henrich PP, Straimer J, Gnädig N, Uhlemann AC, et al. Globally prevalent PfMDR1 mutations modulate Plasmodium falciparum susceptibility to artemisinin-based combination therapies. Nat Commun. 2016. https://doi.org/10.1038/ncomms11553 PMID: 27189525

31. Mbaye A, Dieye B, Ndiaye YD, Bei AK, Muna A, Deme AB, et al. Selection of N86F184D1246 haplotype of Pfmrd1 gene by artemether-lumefantrine drug pressure on Plasmodium falciparum populations in Senegal. Malar J. 2016. https://doi.org/10.1186/s12936-016-1490-4 PMID: 27562216

32. Diallo MA, Yade MS, Ndiaye YD, Diallo I, Diongue K, Sy SA, et al. Efficacy and safety of artemisinin-based combination therapy and the implications of Pfkelch13 and Pfcoronin molecular markers in treatment failure in Senegal. Sci Rep. 2020. https://doi.org/10.1038/s41598-020-65553-5 PMID: 32483161

33. Dieye Y, Mbengue B, Dagamajalu S, Fall MM, Loke MF, Nguer CM, et al. Cytokine response during non-cerebral and cerebral malaria: Evidence of a failure to control inflammation as a cause of death in African adults. PeerJ. 2016. https://doi.org/10.7717/peerj.1965 PMID: 27168977

34. Diawara S, Madamet M, Kounta MB, Lo G, Wade KA, Nakoulima A, et al. Confirmation of Plasmodium falciparum in vitro resistance to monodesethylamodiaquine and chloroquine in Dakar, Senegal, in 2015. Malar J. 2017.

35. Gendrot M, Wague Gueye M, Tsombeng Foguim F, Madamet M, Wade KA, Bou Kounta M, et al. Modulation of in vitro antimalarial responses by polymorphisms in Plasmodium falciparum ABC transporters (pfmdr1 and pfmdr5). Acta Trop. 2019. https://doi.org/10.1016/j.actatropica.2019.05.020 PMID: 31108084

36. Torrentino-Madamet M, Fall B, Benoit N, Camara C, Amalvict R, Fall M, et al. Limited polymorphisms in k13 gene in Plasmodium falciparum isolates from Dakar, Senegal in 2012–2013. Malaria Journal. 2014. https://doi.org/10.1186/1475-2875-13-472 PMID: 25471113

37. Boussaroque A, Fall B, Madamet M, Camara C, Benoit N, Fall M, et al. Emergence of mutations in the K13 propeller gene of Plasmodium falciparum isolates from Dakar, Senegal, in 2013–2014. Antimicrob Agents Chemother. 2016. https://doi.org/10.1128/AAC.01346-15 PMID: 26503652

38. Madamet M, Kounta MB, Wade KA, Lo G, Diawara S, Fall M, et al. Absence of association between polymorphisms in the K13 gene and the presence of Plasmodium falciparum parasites at day 3 after treatment with artemisinin derivatives in Senegal. Int J Antimicrob Agents. 2017. https://doi.org/10.1016/j.ijantimicag.2017.01.032 PMID: 28450175

39. Fall B, Camara C, Fall M, Nakoulima A, Dionne P, Diatta B, et al. Plasmodium falciparum susceptibility to standard and potential anti-malarial drugs in Dakar, Senegal, during the 2013–2014 malaria season. Malar J. 2015; 14(1):1–7.

40. Ménard D, Khim N, Beghain J, Adegnika A, Shafiul-Alam M, Amodu O, et al. A Worldwide Map of Plasmodium falciparum K13-Propeller Polymorphisms. N Engl J Med. 2016; 374(25):2453–64. https://doi.org/10.1056/NEJMoa1513137 PMID: 27332904

41. Ndiaye D, Dieye B, Ndiaye YD, Tyne D Van, Daniels R, Bei AK, et al. Polymorphism in dhfr/dhps genes, parasite density and ex vivo response to pyrimethamine in plasmodium falciparum malaria parasites in

thies, senegal. Int J Parasitol Drugs Drug Resist. 2013. https://doi.org/10.1016/j.ijpddr.2013.07.001 PMID: 24533303

42. Bereczky S, Mårtensson A, Gil JP, Färnert A. Short report: Rapid DNA extraction from archive blood spots on filter paper for genotyping of Plasmodium falciparum. Am J Trop Med Hyg. 2005. PMID: 15772315

43. Escobar C, Pateira S, Lobo E, Lobo L, Teodosio R, Dias F, et al. Polymorphisms in Plasmodium falciparum K13-propeller in angola and mozambique after the introduction of the ACTs. PLoS One. 2015; 10 (3). https://doi.org/10.1371/journal.pone.0119215 PMID: 25789669

44. Ménard D, Khim N, Beghain J, Adegnika AA, Shafiul-Alam M, Amodu O, et al. A worldwide map of Plasmodium falciparum K13-propeller polymorphisms. N Engl J Med. 2016. https://doi.org/10.1056/NEJMoa1513137 PMID: 27332904

45. WWARN. Plasmodium falciparum and artemisinin combination therapies. 2020.

46. Asua V, Vinden J, Conrad MD, Legac J, Kigozi SP, Kamya MR, et al. Changing molecular markers of antimalarial drug sensitivity across Uganda. Antimicrob Agents Chemother. 2019. https://doi.org/10.1128/AAC.01818-18 PMID: 30559133

47. Matrevi SA, Opoku-Agyeman P, Quashie NB, Bruku S, Abuaku B, Koram KA, et al. Plasmodium falciparum Kelch Propeller Polymorphisms in Clinical Isolates from Ghana from 2007 to 2016. Antimicrob Agents Chemother. 2019. https://doi.org/10.1128/AAC.00802-19 PMID: 31427297

48. Kamau E, Campino S, Amenga-Etego L, Drury E, Ishengoma D, Johnson K, et al. K13-propeller polymorphisms in plasmodium falciparum parasites from sub-saharan Africa. J Infect Dis. 2015. https://doi.org/10.1093/infdis/jiu608 PMID: 25367300

49. Bretscher MT, Dahal P, Griffin J, Stepniewska K, Bassat Q, Baudin E, et al. The duration of chemoprophylaxis against malaria after treatment with artesunate-amodiaquine and artemether-lumefantrine and the effects of pfmdr1 86Y and pfcrt 76T: A meta-analysis of individual patient data. BMC Med. 2020. https://doi.org/10.1186/s12916-020-1494-3 PMID: 32098634

50. Okell LC, Reiter LM, Ebbe LS, Baraka V, Bisanzio D, Watson OJ, et al. Emerging implications of policies on malaria treatment: Genetic changes in the Pfmdr-1 gene affecting susceptibility to artemether–lumefantrine and artesunate–amodiaquine in Africa. BMJ Glob Heal. 2018. https://doi.org/10.1136/bmjgh-2018-000999 PMID: 30397515

51. Van Tyne D, Dieye B, Valim C, Daniels RF, Sène PD, Lukens AK, et al. Changes in drug sensitivity and anti-malarial drug resistance mutations over time among Plasmodium falciparum parasites in Senegal. Malar J. 2013. https://doi.org/10.1186/1475-2875-12-441 PMID: 24314037

52. Ndiaye D, Patel V, Demas A, LeRoux M, Ndir O, Mboup S, et al. Short report: A non-radioactive DAPI-based high-throughput in vitro assay to assess Plasmodium falciparum responsiveness to antimalarials —Increased sensitivity of P. falciparum to chloroquine in senegal. Am J Trop Med Hyg. 2010.

53. Ly O, El Hadji Omar Gueye P, Deme AB, Dieng T, Badiane AS, Ahouidi AD, et al. Evolution of the pfcrt T76 and pfmdr1 Y86 markers and chloroquine susceptibility 8 years after cessation of chloroquine use in Pikine, Senegal. Parasitol Res. 2012; 111(4):1541–6. https://doi.org/10.1007/s00436-012-2994-7 PMID: 22706959

54. Nwakanma DC, Duffy CW, Amambua-Ngwa A, Oriero EC, Bojang KA, Pinder M, et al. Changes in malaria parasite drug resistance in an endemic population over a 25-year period with resulting genomic evidence of selection. J Infect Dis. 2014.

55. Xu C, Wei Q, Yin K, Sun H, Li J, Xiao T, et al. Surveillance of Antimalarial Resistance Pfcrt, Pfmdr1, and Pfkelch13 Polymorphisms in African Plasmodium falciparum imported to Shandong Province, China. Sci Rep. 2018.

56. Pillai DR, Lau R, Khairnar K, Lepore R, Via A, Staines HM, et al. Artemether resistance in vitro is linked to mutations in PfATP6 that also interact with mutations in PfMDR1 in travellers returning with Plasmodium falciparum infections. Malar J. 2012. https://doi.org/10.1186/1475-2875-11-131 PMID: 22540925

57. Taylor AR, Flegg JA, Holmes CC, Guérin PJ, Sibley CH, Conrad MD, et al. Artemether-lumefantrine and dihydroartemisinin-piperaquine exert inverse selective pressure on plasmodium falciparum drug sensitivity-associated haplotypes in Uganda. Open Forum Infect Dis. 2017. https://doi.org/10.1093/ofid/ofw229 PMID: 28480232

58. Muiruri P, Juma DW, Ingasia LA, Chebon LJ, Opot B, Ngalah BS, et al. Selective sweeps and genetic lineages of Plasmodium falciparum multi-drug resistance (pfmdr1) gene in Kenya. Malar J. 2018. https://doi.org/10.1186/s12936-018-2534-8 PMID: 30376843

59. Kavishe RA, Paulo P, Kaaya RD, Kalinga A, Van Zwetselaar M, Chilongola J, et al. Surveillance of artemether-lumefantrine associated Plasmodium falciparum multidrug resistance protein-1 gene polymorphisms in Tanzania. Malar J. 2014. https://doi.org/10.1186/1475-2875-13-264 PMID: 25007802

60. Happi CT, Gbotosho GO, Folarin OA, Sowunmi A, Hudson T, O'Neil M, et al. Selection of Plasmodium falciparum multidrug resistance gene 1 alleles in asexual stages and gametocytes by artemether-lumefantrine in nigerian children with uncomplicated falciparum malaria. Antimicrob Agents Chemother. 2009. https://doi.org/10.1128/AAC.00968-08 PMID: 19075074

61. Malmberg M, Ferreira PE, Tarning J, Ursing J, Ngasala B, Björkman A, et al. Plasmodium falciparum drug resistance phenotype as assessed by patient antimalarial drug levels and its association with pfmdr1 polymorphisms. J Infect Dis. 2013. https://doi.org/10.1093/infdis/jis747 PMID: 23225895

62. Baliraine FN, Rosenthal PJ. Prolonged selection of pfmdr1 polymorphisms after treatment of falciparum malaria with artemether-lumefantrine in Uganda. J Infect Dis. 2011. https://doi.org/10.1093/infdis/jir486 PMID: 21881128

63. Nag S, Dalgaard MD, Kofoed PE, Ursing J, Crespo M, Andersen LOB, et al. High throughput resistance profiling of Plasmodium falciparum infections based on custom dual indexing and Illumina next generation sequencing-technology. Sci Rep. 2017. https://doi.org/10.1038/s41598-017-02724-x PMID: 28546554