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Increased *pfmdr*1 copy number and sequence polymorphisms in *Plasmodium falciparum* isolates from Sudanese malaria patients treated with artemether-lumefantrine

*Running Title: pfmdr*1 copy number variation and AL efficacy in Sudan

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ABSTRACT

Molecular markers for surveillance of *Plasmodium falciparum* resistance to current antimalarials are sorely needed. A 28-day efficacy study of artemether-lumefantrine in eastern Sudan identified 5 treatment failures among 100 evaluable patients; nine further individuals were parasite positive by PCR during follow-up. Polymorphisms in *pfatpase6* and *pfmdr1* were evaluated by DNA sequencing. One individual carried parasites with a novel *pfmdr1* polymorphism (F1044L). *pfmdr1* gene amplification in parasites prior to treatment occurred in three individuals who had recurrent infection during follow-up.

Abstract **79 words**

Text **1,509 words**

Artemisinin combination therapies (ACT) are strongly recommended for treating uncomplicated falciparum malaria (33). The most widely adopted of these combinations in sub-Saharan Africa is artemether-lumefantrine (AL) (32). The recent emergence of resistance to artemisinin on the Thai-Cambodian border (23, 5) establishes an urgent need for validated molecular markers of resistance to ACT in general, and to AL in particular.

Evidence from *in vitro* and *in vivo* studies has suggested that polymorphisms in the *pfatpase6* locus, encoding *P. falciparum* SERCA (8, 16, 17, 28, 30), and in the *pfmdr1* locus, encoding the parasite multi-drug resistance transporter Pgh-1 (6, 7, 24, 25), may modulate plasmodium sensitivity to artemisinins (4, 12, 14, 26). Studies in Thailand show that copy number variation (CNV) of the *pfmdr*1 locus is associated with reduced *in vivo* and *in vitro* sensitivity to both mefloquine and AL (24, 25, 34). CNV has not been linked to treatment outcomes with AL in Africa (4, 14) although increased *pfmdr1* copy number has been observed in isolates from Kenya and Gabon (13, 29), and confirmed in a few case studies of travellers returning from west Africa, mostly after mefloquine use (11, 35).

In Sudan a 6-dose course of AL is currently recommended as second-line treatment for uncomplicated falciparum malaria (18), and has reported *in vivo* efficacy of over 90% (9, 20, 22). In the present study we analyse the sequence of *pfatpase6* and *pfmdr*1 alleles, and test for CNV at *pfmdr1*, in parasites before and after AL treatment for uncomplicated falciparum malaria in eastern Sudan.

Patients were recruited from among those presenting with fever or history of fever to clinics in the villages Asar, Daraweesh and Abu Adam near Gedaref town, and in a refugee camp in New-Halfa, an

irrigated area 150km from Gedaref. Inclusion criteria were a positive smear for *P. falciparum* mono-infection, a parasite count of 1,000 – 100,000 asexual parasites/µl (Gedaref), or between 2,000 – 200,000 asexual parasites/µl (New-Halfa), an axillary temperature ≥37.5°C, weight >10kg (Gedaref) or > 5kg (New-Halfa). Pregnant women, patients with other underlying disease or with signs of severe malaria (36) were excluded. Clinical assessment was performed on recruitment (D0) and days 1, 2, 3, 7, 14, 21 and 28. Participants were treated with six doses of AL (20mg artemether/120mg lumefantrine tablets; Novartis). For adults, 4 tablets twice daily were administered; doses were adjusted according to weight for children under 35kg. The first dose each day was observed, the second dose was self-administered. Patients were advised to eat fatty food or milk before each dose. Giemsa-stained thick blood films were prepared and examined by microscopists on each day and a blood spot was collected onto glass fibre membranes for DNA analysis.

DNA extraction utilised a modified Chelex® method (3). Amplification of both genes was attempted for all pre-treatment (D0) samples. Amplification of *pfmdr*1 fragment 1 (6) was attempted for all post-treatment samples from day 14 and later. PCR-positive post-treatment samples were further analysed for other *pfmdr*1 regions and the *pfatpase*6 gene, using previously described methods for nested PCR methods and DNA sequencing (14, 19). Gene copy number was analysed by a duplex dual-labelled probe qPCR assay (24); two independent experiments were performed and in each experiment each isolate was tested in triplicate. Control DNA from *P. falciparum* lines 3D7 and HB3 (1 copy of *pfmdr*1 each) and Dd2 (2 copies of *pfmdr*1) were run in parallel in each experiment. A sample was considered evaluable if it produced a duplex fluorescent signal in at least two replicates in each of the two experiments. Recrudescence was distinguished from re-infection by *pfmsp*-1 and *pfmsp*-2 genotyping (27).

All participants received an information sheet in English and Arabic and provided signed written informed consent. Ethical approval was obtained from the Tropical Medicine Research Institute Ethics Review Committee, and the London School of Hygiene and Tropical Medicine Ethics Committee. The study was registered as a clinical trial (ID: NCT00440752).

106 patients were recruited and 100 (94.3%) completed the follow-up. Mean age of recruits was similar in the two sites, being 16.7 years (95% C.I. 13.0 – 20.4) in Gedaref and 20.9 years (95% C.I. 15.8 – 26.0) in New-Halfa (P = 0.19; 2-tailed Student's t-test). By per protocol analysis, 95 patients (95%) displayed adequate clinical and parasitological response, with 5 individuals failing treatment. The PCR-corrected estimate of therapeutic efficacy was 98%, as three recurrent infections displayed different *msp*-1 and *msp*-2 genotypes from the original infection. Eleven individuals were positive by *pfmdr1* PCR on day 14; three of these remained positive and a further one and two individuals became positive on day 21 and 28 respectively, making a total of 14 recurrent infections identified by PCR; all five individuals identified as treatment failures by microscopy were PCR positive.

Codons 225 - 423 and 470 – 906 of the *pfatpase*6 gene (52% of the locus) were successfully sequenced in 78 and 87 pre-treatment isolates, respectively. 569K occurred in 49.4% of isolates. The synonymous polymorphism nt2694 T-A was detected in 59.3% of these isolates. A novel non-synonymous change (D845N) was detected in 2 isolates. Other previously reported polymorphisms were also observed (Table 1). Three D14, one D21 and two D28 isolates were successfully sequenced, and *pfatpase6* alleles were compared with those present in the same individual prior to treatment

(Table 2). Considerable diversity in this locus is confirmed in this seasonal low transmission setting, but the six evaluable recurrent infections were not associated with particular *pfatpase* genotypes.

A novel polymorphism, F1044L, was observed in *pfmdr*1 in a single multi-clonal pre-treatment isolate; parasites with wild-type sequence at this codon were also present. All isolates, pre- and post-treatment, carried the wild-type amino acids Ser and Asn at codons 1034 and 1042, respectively, in *pfmdr*1. Haplotypes of *pfmdr1* were constructed at codons 86, 184 and 1246 for all isolates with complete data; four isolates with unambiguous mixtures of two haplotypes were each counted as harbouring both. The haplotype YFD was the most common prior to treatment, occurring in 67.4% of the 89 evaluable samples with unambiguous haplotypes, followed by YYD (15.7%), NYD (7.9%), NFD (5.6%), YYY (4.5%), YFY (2.2%) and NFY (1.1%). In contrast, NFD was relatively more abundant in post-treatment isolates (Table 3), being found alone or mixed in five of the fourteen post-treatment isolates (odds ratio 9.33; 95% C.I. 1.72 - 48.2; Fisher's exact $P = 0.004$).

Seventy-four pre-treatment and 14 post-treatment isolates were investigated for evidence of *pfmdr*1 amplification. Copy number estimates of 1.03, 0.81 and 2.19 were obtained for 3D7, HB3 and Dd2. Reliable estimates of *pfmdr*1 copy number from at least two independent experiments were obtained for 57 pre-treatment and 2 post-treatment isolates. The average copy number in pre-treatment isolates was 1.24 (range 0.73 – 2.33; 95% CI; 1.16 – 1.32); *pfmdr1* copy number estimates above 1.8 in at least two independent experiments were obtained for three pre-treatment isolates (Figure 1). Each of these individuals also had recurrent parasitaemia during follow-up; one case was microscopy positive on day 14 and typed by *pfmsp2* genotyping as a recrudescence, whereas the remaining two cases were classified as ACPR but positive by PCR on day 14. There was thus an association between

carriage of parasites with amplified *pfmdr*1 copy number, prior to treatment, and recurrent parasitaemia after AL (Fisher's exact test, P = 0.011). Two post-treatment isolates were successfully tested and both of these carried one copy of the *pfmdr*1 gene. Interestingly, *pfmdr*1 amplified isolates in this study carried the 86Y allele instead of the N86 seen in South East Asia (Table 3). This observation is consistent with other African reports (13, 29; Maja Malmberg, Malaria Research Lab, Department of Medicine, Karolinska Institutet, Stockholm, Sweden; pers. comm.).

The NFD allele is suggested to appear in re-infecting rather than recrudescing parasites following AL treatment (4, 12, 14, 26). Consistent with this view, two of the clinical failures in this study were classified as new infections by *msp*-1/2 genotyping. These were late failures from New-Halfa where the irrigation canals provide a stable mosquito habitat. In contrast, some earlier cases of parasite recurrence were identified by PCR in Gedaref where the possibility of re-infection is low. The observation of a new substitution at codon Phe1044Leu, and the amplification of *pfmdr*1, suggests diversification of this locus may be occurring. However, as the number of observations in our study was small, these preliminary findings need to be confirmed in larger studies.

AL proved efficacious for treating uncomplicated malaria caused by *P. falciparum* in this study across two sites in eastern Sudan. We identified sub-patent parasitaemia in 10 patients with ACPR, but cannot rule out that gametocytes of *P. falciparum* were the origin of this DNA in at least some of these individuals. Selection by AL for genotypes at the *pfatpase6* locus was not observed, but evidence was found of selection by AL for the *pfmdr1* haplotype NFD in recurrent parasitaemia as early as D14 after treatment. We present the first evidence from an African efficacy study that amplification of the *pfmdr1* locus may contribute to recurrent *P. falciparum* parasitaemia following AL

therapy. This association needs to be confirmed in larger studies, particularly as we lack locus amplification data for post-treatment isolates. There is published evidence that injectable artemether mono-therapy has been in use by medical practitioners in northern Sudan (1, 10, 21), and this may have led to selection of parasites carrying this gene amplification (15, 31); no such amplification was observed among 24 isolates collected in 1989 (2). Continual surveillance of *pfmdr1* and other loci implicated in antimalarial treatment response is justified as large-scale use of ACT continues in sub-Saharan Africa.

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References

1. ADAM I, ELWASILA E, MOHAMMED ALI DA, ELANSARI E, ELBASHIR MI. 2004. Artemether in the treatment of falciparum malaria during pregnancy in eastern Sudan. *Trans R Soc Trop Med Hyg.* **98:** 509-513.
2. BABIKER HA, CREASEY AM, BAYOUMI RA, WALLIKER D, ARNOT DE. 1991. Genetic diversity of Plasmodium falciparum in a village in eastern Sudan. 2. Drug resistance, molecular karyotypes and the mdr1 genotype of recent isolates. Trans R Soc Trop Med Hyg. 85: 578-583.
3. DLAMINI, S. V., BESHIR, K. & SUTHERLAND, C. J. 2010. Markers of anti-malarial drug resistance in Plasmodium falciparum isolates from Swaziland: identification of pfmdr1-86F in natural parasite isolates. Malar J, 9, 68.
4. DOKOMAJILAR, C., NSOBYA, S. L., GREENHOUSE, B., ROSENTHAL, P. J. & DORSEY, G. 2006. Selection of Plasmodium falciparum pfmdr1 alleles following therapy with artemether-lumefantrine in an area of Uganda where malaria is highly endemic. Antimicrob Agents Chemother, 50, 1893-5.
5. DONDORP, A. M., NOSTEN, F., YI, P., DAS, D., PHYO, A. P., TARNING, J., LWIN, K. M., ARIEY, F., HANPITHAKPONG, W., LEE, S. J., RINGWALD, P., SILAMUT, K., IMWONG, M., CHOTIVANICH, K., LIM, P., HERDMAN, T., AN, S. S., YEUNG, S., SINGHASIVANON, P., DAY, N. P., LINDEGARDH, N., SOCHEAT, D. & WHITE, N. J. 2009. Artemisinin resistance in Plasmodium falciparum malaria. *N Engl J Med,* **361,** 455-67.
6. DURAISINGH, M. T., JONES, P., SAMBOU, I., VON SEIDLEIN, L., PINDER, M. & WARHURST, D. C. 2000a. The tyrosine-86 allele of the pfmdr1 gene of Plasmodium falciparum is associated with increased sensitivity to the anti-malarials mefloquine and artemisinin. Mol Biochem Parasitol, 108, 13-23.
7. DURAISINGH, M. T., ROPER, C., WALLIKER, D. & WARHURST, D. C. 2000b. Increased sensitivity to the antimalarials mefloquine and artemisinin is conferred by mutations in the pfmdr1 gene of Plasmodium falciparum. Mol Microbiol, 36, 955-61.
8. ECKSTEIN-LUDWIG, U., WEBB, R. J., VAN GOETHEM, I. D., EAST, J. M., LEE, A. G., KIMURA, M., O'NEILL, P. M., BRAY, P. G., WARD, S. A. & KRISHNA, S. 2003. Artemisinins target the SERCA of Plasmodium falciparum. Nature, 424, 957-61.
9. ELAMIN, S. B., AWAD, A. I., ELTAYEB, I. B., ELMARDI, K. A., HASSAN, A. H., MOHAMED, A. O., MALIK, E. M. & MOHAMAD, T. A. 2010. Descriptive study on the efficacy of artemether-lumefantrine in the treatment of uncomplicated Plasmodium falciparum malaria in Sudan. Eur J Clin Pharmacol, 66, 231-7.
10. ELMARDI, K. A., NOOR, A. M., GITHINJI, S., ABDELGADIR, T. M., MALIK, E. M., SNOW, R. W. 2011. Self-reported fever, treatment actions and malaria infection prevalence in the northern states of Sudan. Malaria Journal, 10, 128.
11. GAY, F, BINET MH, BUSTOS MD, ROUVEIX B, DANIS M, ROY C, GENTILINI M. 1990. Mefloquine failure in child contracting falciparum malaria in West Africa. *Lancet* **335:** 120–121.
12. HASTINGS, I. M. & WARD, S. A. 2005. Coartem (artemether-lumefantrine) in Africa: the beginning of the end? J Infect Dis, 192, 1303-4; author reply 1304-5.
13. HOLMGREN G, BJORKMAN A, GIL JP. 2006. Amodiaquine resistance is not related to rare findings of *pfmdr1* gene amplifications in Kenya. *Trop Med Int Health*. **11:** 1808 - 112.
14. HUMPHREYS, G. S., MERINOPOULOS, I., AHMED, J., WHITTY, C. J., MUTABINGWA, T. K., SUTHERLAND, C. J. & HALLETT, R. L. 2007. Amodiaquine and artemether-lumefantrine select distinct alleles of the Plasmodium falciparum mdr1 gene in Tanzanian children treated for uncomplicated malaria. Antimicrob Agents Chemother, 51, 991-7.
15. IMWONG, M., DONDORP, A. M., NOSTEN, F., YI, P., MUNGTHIN, M., HANCHANA, S., DAS, D., PHYO, A. P., LWIN, K. M., PUKRITTAYAKAMEE, S., LEE, S. J., SAISUNG, S., KOECHAROEN, K., NGUON, C., DAY, N. P., SOCHEAT, D. & WHITE, N. J. 2010. Exploring the contribution of candidate genes to artemisinin resistance in Plasmodium falciparum. Antimicrob Agents Chemother, 54, 2886-92.
16. JAMBOU, R., LEGRAND, E., NIANG, M., KHIM, N., LIM, P., VOLNEY, B., EKALA, M. T., BOUCHIER, C., ESTERRE, P., FANDEUR, T. & MERCEREAU-PUIJALON, O. 2005. Resistance of Plasmodium falciparum field isolates to in-vitro artemether and point mutations of the SERCA-type PfATPase6. Lancet, 366, 1960-3.
17. JAMBOU, R., MARTINELLI, A., PINTO, J., GRIBALDO, S., LEGRAND, E., NIANG, M., KIM, N., PHARATH, L., VOLNAY, B., EKALA, M. T., BOUCHIER, C., FANDEUR, T., BERZOSA, P., BENITO, A., FERREIRA, I. D., FERREIRA, C., VIEIRA, P. P., ALECRIM, M. G., MERCEREAU-PUIJALON, O. & CRAVO, P. 2010. Geographic structuring of the Plasmodium falciparum sarco(endo)plasmic reticulum Ca2+ ATPase (PfSERCA) gene diversity. PLoS One, 5, e9424.
18. MALIK EM, S. O. 2004. Malaria in Sudan: past, present and the future. *Gezira Journal of Health Sciences* **1:** 47-53.

19. MENEGON, M., SANNELLA, A. R., MAJORI, G. & SEVERINI, C. 2008. Detection of novel point mutations in the Plasmodium falciparum ATPase6 candidate gene for resistance to artemisinins. Parasitol Int, 57, 233-5.
20. MOHAMED, A. O., ELTAIB, E. H., AHMED, O. A., ELAMIN, S. B. & MALIK, E. M. 2006. The efficacies of artesunate-sulfadoxine-pyrimethamine and artemether-lumefantrine in the treatment of uncomplicated, Plasmodium falciparum malaria, in an area of low transmission in central Sudan. Ann Trop Med Parasitol, 100, 5-10.
21. MOHAMMED-ALI, A. R., KHEIR, M. M. & ADAM, I. 2006. Intramuscular artemether in the treatment of uncomplicated Plasmodium falciparum malaria in Sudanese patients. Saudi Med J, 27, 122-3.
22. MUKHTAR, E. A., GADALLA, N. B., EL-ZAKI, S. E., MUKHTAR, I., MANSOUR, F. A., BABIKER, A. & EL-SAYED, B. B. 2007. A comparative study on the efficacy of artesunate plus sulphadoxine/pyrimethamine versus artemether-lumefantrine in eastern Sudan. *Malar J*, **6**: 92.
23. NOEDL, H., SE, Y., SCHAECHER, K., SMITH, B. L., SOCHEAT, D. & FUKUDA, M. M. 2008. Evidence of artemisinin-resistant malaria in western Cambodia. *New Engl J Med* **359:** 2619 – 2620.
24. PRICE, R. N., UHLEMANN, A. C., BROCKMAN, A., MCGREADY, R., ASHLEY, E., PHAIPUN, L., PATEL, R., LAING, K., LOOAREESUWAN, S., WHITE, N. J., NOSTEN, F. & KRISHNA, S. 2004. Mefloquine resistance in Plasmodium falciparum and increased pfmdr1 gene copy number. Lancet, 364, 438-47.
25. PRICE RN, UHLEMANN A-C, VAQN VUGT M, BROCKMAN A, HUTAGALUNG R, NAIR S, NASH D, SINGHASIVANON P, ANDERSON TJC, KRISHNA S, WHITE NJ, NOSTEN F. 2006. Molecular and pharmacological determinants of the therapeutic response to artemether-lumefantrine in multidrug-resistant Plasmodium falciparum malaria Clin. Infect. Dis, 42: 1570-1577.
26. SISOWATH, C., STROMBERG, J., MARTENSSON, A., MSELLEM, M., OBONDO, C., BJORKMAN, A. & GIL, J. P. 2005. In vivo selection of Plasmodium falciparum pfmdr1 86N coding alleles by artemether-lumefantrine (Coartem). J Infect Dis, 191, 1014-7.
27. SNOUNOU, G., ZHU, X., SIRIPOON, N., JARRA, W., THAITHONG, S., BROWN, K. N. & VIRIYAKOSOL, S. 1999. Biased distribution of msp1 and msp2 allelic variants in Plasmodium falciparum populations in Thailand. Trans R Soc Trop Med Hyg, 93, 369-74.
28. UHLEMANN, A. C., CAMERON, A., ECKSTEIN-LUDWIG, U., FISCHBARG, J., ISEROVICH, P., ZUNIGA, F. A., EAST, M., LEE, A., BRADY, L., HAYNES, R. K. & KRISHNA, S. 2005. A single amino acid residue can determine the sensitivity of SERCAs to artemisinins. Nat Struct Mol Biol, 12, 628-9.
29. UHLEMANN, A. C., RAMHARTER, M., LELL, B., KREMSNER, P. G., KRISHNA, S. 2005. Amplification of Plasmodium falciparum multidrug resistance gene 1 in isolates from Gabon. Journal of Infectious Diseases, 192, 1830-5.
30. VALDERRAMOS, S. G., SCANFELD, D., UHLEMANN, A. C., FIDOCK, D. A. & KRISHNA, S. 2010. Investigations into the role of the Plasmodium falciparum SERCA (PfATP6) L263E mutation in artemisinin action and resistance. Antimicrob Agents Chemother, 54, 3842-52.
31. VINAYAK, S., ALAM, M. T., SEM, R., SHAH, N. K., SUSANTI, A. I., LIM, P., MUTH, S., MAGUIRE, J. D., ROGERS, W. O., FANDEUR, T., BARNWELL, J. W., ESCALANTE, A. A., WONGSRICHANALAI, C., ARIEY, F., MESHNICK, S. R. & UDHAYAKUMAR, V. 2010. Multiple genetic backgrounds of the amplified Plasmodium falciparum multidrug resistance (pfmdr1) gene and selective sweep of 184F mutation in Cambodia. Journal of Infectious Diseases, 201, 1551-1560.
32. VON SEIDLEIN, L., BOJANG, K., JONES, P., JAFFAR, S., PINDER, M., OBARO, S., DOHERTY, T., HAYWOOD, M., SNOUNOU, G., GEMPERLI, B., GATHMANN, I., ROYCE, C., MCADAM, K. & GREENWOOD, B. 1998. A randomized controlled trial of artemether/benflumetol, a new antimalarial and pyrimethamine/sulfadoxine in the treatment of uncomplicated falciparum malaria in African children. Am J Trop Med Hyg, 58, 638-44.
33. WHITE, N. J. 1999. Delaying antimalarial drug resistance with combination chemotherapy. Parassitologia, 41, 301-8.
34. WILSON, C. M., VOLKMAN, S. K., THAITHONG, S., MARTIN, R. K., KYLE, D. E., MILHOUS, W. K. & WIRTH, D. F. 1993. Amplification of pfmdr 1 associated with mefloquine and halofantrine resistance in Plasmodium falciparum from Thailand. Mol Biochem Parasitol, 57, 151-60.
35. WITKOWSKI B, IRIART X, SOH PN, MENARD S, ALVAREZ M, NANEIX-LAROCHE V, MARCHOU B, MAGNAVAL JF, BENOIT-VICAL F, BERRY A. 2010. *pfmdr1* amplification associated with clinical resistance to mefloquine in West Africa: implications for efficacy of artemisinin combination therapies. *J Clin Microbiol*. **48:** 3797-9.
36. WORLD HEALTH ORGANIZATION. 2000. Severe falciparum malaria. *Trans R Soc Trop Med Hyg* **94(Suppl 1):** S1–S90.

37. **Table 1. Diversity of *pfatpase6* at recruitment.**

Study site	L402**V**		N569**K**		G639**D**		E643**Q**		I723**V**		I735**M**		D845**N**	
	L	V	N	K	G	D	E	Q	I	V	I	M	D	N
New Halfa	35	4	22	20	41	1	41	1	42	0	39	3	40	2
Gedaref	35	4	22	23	44	1	45	0	42	3	42	1	39	0
Total	**70**	**8**	**44**	**43**	**85**	**2**	**86**	**1**	**84**	**3**	**81**	**4**	**79**	**2**

Amplification primers

nest 1: ATP6_FOR1 5'-AATAAAACTCCCGCTGATGC-3; ATP6_REV1 5'-ATCCTTCTTCTCCATCATCC-3'

nest 2: ATP6_FOR1; ATP6_REV2 5'-CGTTAAAGCTTCAACATTTCC-3' (800bp).

Changed amino acid in bold; L; leucine, V; valine, N; asparagine, K; lysine, G; glycine, D; aspartic acid, E; glutamic acid, Q; glutamine, I; isoleucine, M;methionine.

The E431K polymorphism was not investigated in this study.

Table 2. Comparison of *pfatpase*-6 in pre-treatment and post-treatment isolates

Sample	Day-0	Day-14	Day-21	Day-28	Treatment out come
9	L**K**GEIID**A**		-NGEIID**A****		LPF Day-21
19	LNGEIID**A**			-NGEI**M**DT	LCF Day-28
56 *	-**K**GEIIDT	-**K**GEIIDT/**A****			ACPR
59	-NGEIIDT	LNGEIIDT			LPF Day-14
74 *	**VK**GEIIDT	-**K**GEIIDT		-**K**GEIID**A**	ACPR

Amino acids L402**V**, N569**K**, G639**D**, E643Q, I723**V**, I735**M**, D845**N** & nucleotide T2694**A**

Changed amino acid in bold, T; threonine, A; alanine.

ACPR: adequate clinical and parasitological response; LPF: late parasitological failure; LCF: late clinical failure

*PCR positive on follow up, not a clinical failure

**Mixed at position T2964A

- DNA not available

285

286

287 **Table 3. Longitudinal analysis of *pfmdr1 h*aplotypes in 14 patients with recurrent parasitaemia**

Site	Patient ID	Day 0 haplotype	Day 0 CNV status	Day 14 haplotype	Day 28 haplotype	Clinical outcome
New Halfa	9	YFD	wt		NFD*	LPF
	19	YYD	wt		NFD	LCF
Gedaref	56	YFD	wt	NYD		ACPR
	59	YFD	2 copies	NFD		LPF
	61	YFD	wt	YFD		ACPR
	62	YFD	wt	NFD		LPF
	63	YFY	2 copies	NFD		ACPR
	64	NYD	wt	NYY		LCF
	65	YFD	wt	NYD		ACPR
	68	YYD	wt	YFD		ACPR
	73	YFD	wt	YFD		ACPR
	74	YFD	wt		YFD	ACPR
	75	YFD	2 copies	NYD		ACPR
	82	YFD	-		NYD	ACPR

288 *Day 21

289 Individuals shown include those with sub-microscopic PCR-positive parasitaemia.

290 Wt: wild-type with respect to *pfmdr1* CNV.

291 -: amplification assay unsuccessful

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293

294

FIGURE LEGENDS

Figure 1. Frequency distribution of *pfmdr1* copy number estimates.

Estimates of *pfmdr1* locus copy numbers obtained from 55 pre-treatment isolates with complete follow-up data are grouped in bins of 0.1 copy units. The values shown represent the mean of at least two independent experiments; each DNA sample in each experiment was run in duplicate. Red data are pre-treatment parasite isolates from patients without subsequent recurrent parasitaemia. Green data are pre-treatment parasite isolates from patients with later recurrent parasitaemia by microscopy and/or PCR. Isolates with copy number estimates of 1.8 and above were considered true duplications.