

Prevalence of *Pfmdr 1* N86Y and Y184F Alleles is Associated with Recurrent Parasitemia following Treatment of Uncomplicated Malaria with Artemether-Lumefantrine in Nigerian Patients

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ABSTRACT

We investigated and compared genetic variations in *Plasmodium falciparum* multidrug resistance 1 gene (*Pfmdr 1*) in patients showing good therapeutic response (GTR) and artemisinin resistance (AR) following artemether-lumefantrine (AL) treatment of uncomplicated malaria in Nigeria. Some 150 malaria patients were subjected to AL treatment and therapeutic efficacy was monitored for 28 days. Parasite genomic DNA was isolated followed by nested polymerase chain reaction (PCR). Genotyping of *Pfmdr 1* gene for specific genetic variants: N86Y, Y184F, S1034C and N1042D were done using PCR-restriction fragment length polymorphism (PCR-RFLP). Out of 121 patients that were *P. falciparum* positive, 46 % (56) and 54 % (65) showed good therapeutic response and artemisinin resistance respectively, with 5.4 % and 98.3 % being mutated in the GTR and AR group respectively. The most prevalent mutations were Y184F (44.1 %) and N86Y (40.7 %). There was significant increase ($p < 0.001$) in the prevalence of *Pfmdr 1* mutation in the post treatment compared to the pre-treatment group. Prevalence of *Pfmdr 1* 86Y and 184F alleles is associated with artemisinin resistance and presence of AL drug significantly induced genetic variation in the plasmodial gene.

INTRODUCTION

Malaria is the world's most widely spread parasitic infection with 1-2 million deaths attributable to the disease annually (Guerra *et al.*, 2006; Hay *et al.*, 2004; Snow *et al.*, 2005). Nineteen countries in Africa, including Nigeria, account for 90% of all WHO estimated cases of malaria in 2006 (UK Aid Department for International Development, 2010). More than half of all the estimated world malaria cases occurred in Nigeria and India (Hay *et al.*, 2007). Malaria imposes great burden in terms of pain and trauma suffered by its victims as well as loss in output and cost of treatment (Onwujekwu *et al.*, 2007). Every year, Nigeria loses over 132 billion Naira (over \$1 billion) from

the cost of treatment and absenteeism from work due to malaria attack (Federal Ministry of Health, 2005). *P. falciparum* has also become the predominant malaria specie in many parts of the world (Guerra *et al.*, 2006) and continues to be a major threat to travelers to tropical regions (Mayxay *et al.*, 2004).

There is accumulating evidence to show that the therapeutic efficacy of Chloroquine (CQ) and Sulfadoxine – pyrimethamine (SP), the major drugs for treatment of uncomplicated malaria in Nigeria have deteriorated due to *P. falciparum* resistance (Ezedinachi *et al.*, 1992; Falade *et al.*, 1997; Ekanem *et al.*, 2000). Hence in 2005, Nigerian National Antimalarial Treatment Policy (NNATP) adopted AL as first line treatment of uncomplicated malaria caused by *P. falciparum* in Nigeria (Nosten *et al.* 2007; White, 2008). Antimalarial drug resistance has been associated with specific point mutations in several genes of the *Plasmodium falciparum* (Duraisingh and Cowman, 2005).

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An important gene; *Plasmodium falciparum* multidrug resistant gene 1 (*Pfmdr1*) located on chromosome 5 of the *P. falciparum* genome, which shows similarity to the P-glycoprotein product of the human multidrug resistance gene *mdr*, is strongly associated with resistance to anti-malarial drugs (Rohrbach *et al.*, 2006). The role of genes such as *Plasmodium falciparum* chloroquine resistance transporter (*Pfcr*), *Pfmdr1* and Dihydrofolate reductase (*dhfr*) in antimalarial drug resistance has been studied (Falade *et al.*, 2005). The efficacy and effectiveness of AL as an antimalarial drug has been reported (Falade *et al.*, 2005; Yeka *et al.* 2008; Zurovac *et al.*, 2008). Recent reports from Africa have shown some evidence of clinical and parasitological failure after treatment with AL (Yang *et al.*, 2003; Jambou *et al.*, 2005; Noedl *et al.*, 2010).

Studies have shown that genetic variations like single nucleotide polymorphism (SNPs), mutations and copy number variations (CNV) in such resistance associated genes like *Pfmdr1*, alter the respective protein structural conformation in the *Plasmodium*, hence reducing drug binding or altering molecular transport system leading to parasite surviving the drug effect (Duraisingh and Cowman, 2005). Genetic variations at various positions on the *Pfmdr1* gene (N86Y, Y184F, S1034C, N1042D and D1246Y) have been shown to be associated with resistance to chloroquine, mefloquine, quinine, halofantrine, lumefantrine and artemisinins (Dokomajilar *et al.*, 2006; Sisowath *et al.*, 2005). Recent finding has shown that treatment with the widely advocated AL is associated with selection of newly infecting parasites containing the *Pfmdr1* 86N allele (Sisowath *et al.*, 2005). The Y86N allele has been associated with decrease in vitro sensitivity to artemisinins and lumefantrine (Duraisingh *et al.*, 2000a). In this study we investigated and compared genetic variations in *Plasmodium falciparum* multidrug resistance 1 gene (*Pfmdr 1*) in patients showing good therapeutic response (GTR) and artemisinin resistance (AR) following artemether-lumefantrine (AL) treatment of uncomplicated malaria in Nigeria.

MATERIALS AND METHOD

Study Area and Sampling

The study was conducted between April-November, 2013 in three hospitals: the District Hospital Nsukka, Bishop Shanahan Hospital, Nsukka and Cottage Hospital Ugbuawka, all in Enugu State, South- Eastern Nigeria.

The study area has been described elsewhere^[25]. Out – patients of 6 years and above presenting with symptoms indicating acute uncomplicated malaria with a body temperature of ≥ 37 °C and history of fever in 24 – 48 h preceding presentation were enrolled for the study^[21]. Ethical approval was obtained from the University of Nigeria Teaching -Hospital (UNTH) Health Research Ethics Committee, Enugu. The reference number is NHREC/05/01/2008B-FWA00002458-IRB00002323. Informed consent was obtained from adult participants and from parents for children under their care. Consent was also obtained from patients to publish this information. Patients with history of anti-malarial

use 2 weeks before presentation were excluded. Infants and young children below 6 years old were excluded to reduce non-compliance due to vomiting. In addition, patients with complicated malaria, sickle cell anemia, known allergy to study drug, and HIV positive were equally excluded.

Patient's Treatment

Patients who presented with symptoms such as headache, fever, shivering and vomiting tendency during the past 24 hours to the study hospitals, were examined using thick film microscopy. Their body temperature readings were taken and recorded. The patients received tablets of AL (Coartem[®], Novartis AG, Basel, Switzerland) in the order indicated in Table 1. Each tablet of AL contains 20 mg of artemether and 120 mg of lumefantrine. An easy to fill compliance form with six boxes for ticking at each time drug is taken was designed to help the patients take appropriate dose at the right time, since patients were not in-patients.

All participants were educated on how to tick the compliance form as some were not literate. The compliance form was given to each patient along with drug and was submitted on the next follow up visit on day 3. This was used to monitor patient's compliance.

Table 1: Doses of artemether-lumefantrine drugs for different age ranges and body weight.

Body weight (kg)	Age (years)	Duration (Days / Time)					
		1		2		3	
		0 h	8 h	20h	32h	44h	56h
5 – 14	< 3	1 tab ^a	1 tab	1 tab	1 tab	1 tab	1 tab
15 - 24	> 3 – 8	2 tab	2 tab	2 tab	2 tab	2 tab	2 tab
25 - 34	> 9 – 14	3 tab	3 tab	3 tab	3 tab	3 tab	3 tab
> 34	> 14	4 tab	4 tab	4 tab	4 tab	4 tab	4 tab

Doses of artemether-Lumefantrine administered to each patient group in different age brackets and body weights (kg). The treatments were given at different time intervals (0hr, 8h, 20h, 32h, 44h and 56h) in 3days. ^a = tablet, each tablet of AL contains artemether, 20mg, lumefantrine, 120mg).

DNA Extraction from Blood Samples

P. falciparum genomic DNA was extracted from the blood samples by saponin hemolysis method (Ayogu *et al.*, 2015, Orjih *et al.*, 2008). The purity and quality of the DNA was determined using Eppendorf BioPhotometer Plus (Eppendorf, Germany). The A_{260/280} ratios were between 1.7 – 2.0. The DNA samples were stored at -20 °C until required for analysis.

Plasmodium falciparum Detection and Quantification by Nested PCR and Real time PCR

PCR was used to amplify multiple copies of *Plasmodium* genomic DNA. The PCR (Applied biosystem 2720, Singapore, S/No 272S1132046) was run in two rounds. Round one was run to detect the genus *Plasmodium malaria* using a primer set, RI (rPLU5 and rPLU6) of amplicon size, 1200 base pair (bp) (Table 2). The PCR product from round one was used in round two in the

Real time PCR (Applied biosystem StepOne™, Singapore S/No 271002578) for detection and quantification of *Plasmodium falciparum* species. For the second round PCR, primer set R2, (rFa11 and rFa12) with amplicon size, 205 bp was used (Table 2). The procedures and thermal profile used have been described elsewhere (Ayogu *et al.*, 2015).

Table 2: Mutation sites and the primer sequences used for nested PCR and genotyping of *Pfmdr 1* gene.

Gene	Mutation ^a	Primer sequence ^a
<i>Pfmdr 1</i> N86Y		5'ATGGGTAAAGAGCAGAAAGAG3'
		5'CGTACCAATTCCTGAACCTCAC3'
Y84F		5'CAAGAAGGAAGTAAGTATCCAAAAATGG3'
		5'CTGAAGGCATCTAACATGGATATAGC3'
S1034C		5'CAAGAAGGAAGTAAGTATCCAAAAATGG3'
		5'CTGAAGGCATCTAACATGGATATAGC3'
N1042D		5'TATGTCAAGCGGAGTTTTTGC3'
Primer		
R1	rPLU5	5'CCTGTTGTTGCCTTAAACTTC3'
	rPLU6	5'TTAAAATTGTTGCAGTTAAAC3'
R2	rFa11	5'TTAAACTGGTTGGGAAAACC3'
	rFa12	5'ACACAATGAACCTCAATCATGA3'

Mutation= Polymorphic sites of *Pfmdr 1* gene (N = asparagine, F = phenylalanine, Y = tyrosine, S = serine, C = cysteine and D = aspartate). ^a= primer sequences specific for each mutation.

Classification of Patients into Two Groups: Good Therapeutic Response and Artemisinin Resistance Group

Based on the WHO guideline which states that the working definition of artemisinin resistance is an increase in parasite clearance time as evidenced by $\geq 10\%$ of cases with parasites detectable on day 3 after treatment with an ACT (suspected resistance) (WHO, 2013), patients were grouped into two: those that showed good therapeutic response (GTR) and artemisinin resistance (AL). The two groups of patients were genotyped and the frequency of mutation in the *Pfmdr 1* genes compared.

Genotyping of Parasite Genomic DNA for *Pfmdr 1* Genes

Genotyping was based on the standardized PCR reaction and restriction fragment length polymorphism (RFLP) based on previous protocols (Thompson *et al.*, 2011) with modifications according to available resources. For amplification of the *Pfmdr1* gene coding region, a nested PCR protocol was adopted followed by RFLP.

For the analysis of SNPs in *Pfmdr 1* gene, *P. falciparum* laboratory parasite isolate 3D7 was used as positive controls, while

negative control was gotten from infants below 6 months that has not been exposed to malaria infection. The PCR was run in two rounds: outer and nested PCR, which were done with a primer pair for amplification of codon 86, 184, 1034 and 1042 of *Pfmdr 1* genes where resistance - associated mutations were thought to occur (Table 2).

PCR master mix contained 1:1 TEMPase polymerase (including buffer and dNTP; Thermo Scientific. Germany S/No 00125960) to a final volume of 6.3 μ l, 3.7 μ l of primer for each codon (86, 184, 1034 and 1042) (Table 2) and 2.5 μ l gDNA were transferred into 96 well PCR plate and capped properly. The PCR was run in a thermal cycler in a reaction volume of 12.5 μ l. The thermal profile was 94 °C for 15 min followed by 30 cycles at 94 °C for 30 sec, 55 °C for 50 sec, 72 °C for 90 sec, 72 °C for 5 min; nested PCR 94 °C for 15 min followed by 30 cycles at 94 °C for 30 sec 60 °C for 60 sec 72 °C for 90 sec 1 cycle at 72 °C for 5 min. The PCR was run on a thermal cycler (Applied Biosystem 2720, Singapore, S/No 272S1132046)

SNP Determination at Codon 86, 184, 1034 and 1042 by Restriction Fragment Length Polymorphism

For the PCR-RFLP methodology, the digestive enzymes used were designed to cut the DNA if mutated showing double band but if wild to remain uncut showing a single band. At the end of the round two of the nested PCR amplification of each *Pfmdr1* mutation, RFLP was carried out by adding 17 μ l of enzyme specific for each SNP into 5 μ l of the nested PCR products to a volume of 22 μ l.

The plate was properly capped and placed in an incubator (DNP 9022A, SANFA) for 16 h for normal digest and 45 mins for fast digest at the respective enzymes optimum temperatures according to the manufacturer's instructions (Fermentas, Germany) (Table 3). Restriction enzyme Dra I, Dra II, Hae III and Hin FI were used to digest codon 86, 184, 1034 and 1042 respectively (Table 3).

Table 3: Comparison of frequencies of *Pfmdr 1* mutation in patients with good response against those with treatment failure.

<i>Pfmdr1</i> mutations	Restriction enzyme	Artemisinin resistance	Good therapeutic response
		N = 59 Mutated (%)	N = 56 Mutated (%)
N86Y	Dra I ^a	24 (40.0)	2 (3.6)
Y184F	Dra II ^a	26 (43.9)	1 (1.8)
S1034C	Hae III ^b	9 (15.0)	0 (0)
N1042D	Hin FI ^b	0 (0)	0 (0)
Total		59 (98.3)	3 (5.4)

The distribution of mutations among the patients that responded to treatment and those that had treatment failure. N= Number of patients for each treatment group; Numbers in bracket indicate the percentages for each allelic sites; ^a = Normal Digest; ^b = Fast Digest.

Comparison of Frequencies of *Pfmdr1* Mutation in Pre -and Post-Treatment Groups

In order to check if AL treatment had any effect on the genetic variations observed in the *Pfmdr1* gene, samples were divided into two separate groups: Pre-treatment and post treatment group. The prevalence of the different SNPs observed in pre-treatment group were analyzed and compared to that observed in the post treatment group.

Gel Electrophoresis of Digested Gene

PCR digests were electrophoresed on 2 % agarose gel at 100 volts for 20-30 min. The gel was visualized under ultra-violet (UV) light on a UV Trans-illuminator (VilberLourmart, France). The bands were viewed with UV radiation blocking spectacles (USA).

Statistical Analysis

Data was analyzed using GraphPad Prism version 5.02. Descriptive statistics from the data sets include percentages, means and standard deviations and were used in comparing initial and post treatment clinical and biological characteristics (such as temperature and geometric mean parasitemia). Differences in proportions of treatment outcome and frequency in occurrence of *Pfmdr1* gene mutation in pre-treatment and post treatment groups were analyzed using the Chi-square test or Fisher's exact test. While the differences in parasitic densities of the various groups were analyzed using ANOVA.

Genotyping data at all four *Pfmdr1* loci were combined to determine whether the *Pfmdr1* N86Y-Y184F-S1034C-N1042D allele was present in pre- and post-treatment samples.

RESULTS

A total of 154 patients were sampled, 53 were males while 101 were females. Grouping of 154 sampled patients showed that 31 patients were between 6-12 years of age, 45 were 13-25 years, 30 were 26-40 years whereas 48 were 40 years and above. Temperature readings from the sampled patients were variable but maintained a steady range (35-39 °C) in all the patients during the 28 days follow up, although most of the patients exhibited higher temperatures at day 0.

Detection and Quantification of *Plasmodium falciparum*

The Real time PCR result showed that out of the 154 patients sampled, 121 (78.6 %) were infected with *Plasmodium falciparum* while 33 (21.4 %) were not.

Grouping of Samples Based on Responses to Treatment

The 121 patients were grouped into 2 treatment response groups; Good therapeutic response (GTR) and artemisinin resistance (AR) group. Fifty six (46.3 %) patients that had total parasitemia clearance on days 3 and 7 post treatment without parasitemia recurrence and they were classified under GTR, while 65 (53.7 %) patients with increased parasite clearance time and

those with recurrent parasitemia on days 14 and 28 were classified under AR group. Out of the 65 samples under AR group, 60 (92.3 %) were successfully genotyped while 5 (7.7 %) failed genotyping.

Comparison of Frequency of *Pfmdr1* Gene in Patients with Good Therapeutic Response against Those with Artemisinin Resistance

The genotype result showed that for the GTR group, 3 (5.3 %) and 1 (1.8 %) had N86Y and Y184F mutations respectively. There was no patient with S1034D and N1042D mutations in this group, while in the AR group, 24 (40 %), 26 (43.9 %) and 9 (15 %) had N86Y, Y184F and S1034C mutations respectively (Table 3). There was no patient with N1042D mutation. A total of 59 (98.3 %) out of 60 patients in AR group had one form of mutation or another. The individual mutations in the AR group were not significantly associated with treatment failure ($P > 0.06$) but the cumulative effects of the three mutations were significantly associated with treatment failure ($P > 0.01$).

Patients with Co-mutation

Furthermore, some samples showed co-mutations as they were positive for 2 SNPs. A total of 28.3 % had both N86Y + Y184F, 5.0 % had N86Y + S1034C and 8.3 % of the patients had S1034C + Y184F SNP respectively in the group with treatment failure. However, in the group with good treatment response, only 1.6 % of the patients had co-mutation (N86Y and Y184F) (Table 4). A total of 30.0 % of the patients had N86Y and Y184F form of co-mutation which was the highest in prevalence.

Table 4: Distribution of co-mutation in patients in study area.

Co-mutation	Artemisinin resistance (N = 59) ^a	Good therapeutic response (N = 56)	Total (%)
N86Y + Y184F ^b	17	1	18 (30.0) ^c
N86Y + S1034C	3	0	3 (5.0)
S1034C + Y184F	5	0	5 (8.3)
Total	25	1	26 (21.5)

The frequency and distribution of co-mutation among the patients that responded to treatment and those that had treatment failure. ^a= Number of patients in each treatment group; ^b= Presence of more than one mutation (Co-mutation) in any sampled patient; ^c= Percentage distribution of each co-mutation

Comparison of Frequencies of *Pfmdr1* Mutation in Pre -and Post-Treatment Groups

Prevalence of N86Y SNP increased significantly from 25 to 91.6 %, Y184F SNP from 30.7 to 92.5 % and S1034C SNP from 44.4 to 100.0 % in the pre-treatment to post-treatment groups respectively ($P > 0.001$) (Table 5).

DISCUSSION

Reduced susceptibility to ACT is a major concern in Africa where Artemether-lumefantrine has been adopted as the first line drug for malaria treatment. It has been observed that drug resistance has arisen through acquisition of mutations in drug targets and in drug transporters (Gregson and Powel, 2005). In Nigeria AL has been widely used for the treatment of uncomplicated malaria following its adoption as first line treatment in 2005; Consequently, there have been locally generated reports from Nigeria suggesting reduced responses to and /or emerging resistance to artemisinin combination therapies (Ayogu *et al.*, 2015; Ukwe *et al.*, 2010; Happi *et al.*, 2009). In this study we present the involvement of SNPs in *Pfmdr 1* gene in artemisinin resistance using malaria patients treated with AL therapy in Enugu South East Nigeria, focusing on the difference in the genetic variations in the *Pfmdr 1* gene of patients that had good therapeutic response (GTR) and those that showed artemisinin resistance (AR).

For the purpose of this study, patients were monitored and evaluated on days 0, 3, 7, 14 and 28 because the presence of day 3 parasitemia is the most useful method for assessing artemisinin resistance in mobile patients (Vredan *et al.*, 2013) and it requires less frequent blood sampling (Stepniewska *et al.*, 2010; White, 2011). Over half of the patient population screened in this study had variable mutations at one codon of the *Pfmdr 1* gene or the other. This high prevalence of *Pfmdr 1* mutations suggests the possibility of ancient clones of *P. falciparum* with reduced susceptibility to artemisinin derivatives in the study area. This result also confirms the prevalence of these mutations in the study area.

In classifying study patients into GTR and AR we considered the following; WHO working definition of artemisinin resistance as an increase in parasite clearance time as evidenced by $\geq 10\%$ of cases with parasites detectable on day 3 after treatment with an ACT (WHO, 2013), that AL is known for rapid parasitemia clearance by the quick onset of action of artemether and a sustained parasitemia clearance by the longer acting action of lumefantrine, and finally that artemisinins are highly active against young circulating ring-stage parasite before cytoadherence occurs. The presence of parasitemia on day 3 post infection has been used as an indicator of reduced sensitivity of *P. falciparum* parasite to treatment with artemisinin derivatives^[32]. Consequently, all patients with increased parasite clearance time and hence having presence of parasitemia on day 3 and 7 and those with recurrent parasitemia on day 14 and 28 were all grouped under AS group, while patient with total parasite clearance on day 3 and 7 post treatment without parasitemia recurrence on day 14 and 28 were classified under GTR group.

Analysis of the results done showed that almost all the patients that had artemisinin resistances which were genotyped had one form of *Pfmdr 1* SNP or another with the most common allele being N86Y and Y184F. This is indicative of a gradual gaining of stability of these genotypes in Enugu Nigeria. Comparing this

result with previously reported cases in Nigeria and beyond, Happi *et al.*, 2009 recorded a higher prevalence of *Pfmdr 1* Y184F in their study while Menard *et al.*, 2006 reported a lower prevalence of *Pfmdr 1* N86Y allele. In this study, *Pfmdr 1* S1034C allele was also observed but occurred at a lower frequency compared to *Pfmdr 1* N86Y and Y184F while *Pfmdr 1* N1042D allele was not observed in any of the patients tested. The absence of N1042D allele within the study group could not be explained, however a similar finding has been reported in other studies Dokomajila *et al.*, 2006; Happi *et al.*, 2009 while our result differs from another study carried out in Ghana, where the prevalence of *Pfmdr 1* N1042D allele was found to increase from 2003-2010 (Duah *et al.*, 2013). It is worthy of note that it is the cumulative rather than individual effect of these *Pfmdr 1* SNPs that contributed significantly ($P < 0.001$) to artemether-lumefantrine resistance. On the other hand, the analysis of patients with good therapeutic response resulted in very low frequency of *Pfmdr 1* N86Y and Y184F allele. None of the patients under this category had either S1034C or N1042D SNPs. The occurrence of *Pfmdr1* mutation in this group of patients might be due to incomplete AL treatment before enrolment which possibly had subjected the *Plasmodium* to sub therapeutic dose level of AL. It is also possible that the mutation in *Pfmdr1* gene may not be the only mechanism of AL resistance by *Plasmodium falciparum*.

It was also observed that some patients were positive for more than one mutation in *Pfmdr 1* which was regarded as co-mutation. The prevalence of N86Y+Y184Y mutation was higher than that of N86Y+S1034D and Y184F+S1034C. The prevalence of *Pfmdr 1* co-mutation was higher in the AR group than in the GTR group, even though a few patients who responded to treatment had co-mutation. The prevalence of co-mutation may be due to the fact that in areas of high endemicity of *P. falciparum*, human host are often super infected with multiple clones of the parasite (Smith *et al.*, 1999). *Pfmdr 1* N86Y+Y184F co-mutation significantly contributed to AL resistant ($P < 0.001$). This occurrence of parasite with *Pfmdr 1* co-mutation is a threat to artemisinin therapy.

Evaluating the prevalence of mutations in the *Pfmdr 1* gene at the baseline (day 0) and comparing it with follow up days post treatment, it was observed that there was a significant ($P < 0.001$) increase from pre-treatment to post-treatment prevalence of *Pfmdr 1* N86Y, Y184F and S1034C allele. Our data also revealed that before exposure to AL therapy, most of the *Pfmdr 1* genes were wild type while soon after introduction of AL very few remained wild type while majority of them were mutated. This implies that the presence of the AL induced genetic variations in the *Pfmdr 1* gene. The increase in the prevalence of the *Pfmdr1* N86Y and Y184F alleles that we observed after AL treatment compared to the prevalence in the baseline population could possibly result from *P. falciparum* undergoing genetic/structural changes as a defense mechanism for survival during AL therapy or that these alleles were selected for on a population level but not on an individual level with the recent increase in the use of AL at the study site. It could also be that in AL-treated patients, lumefantrine

might have selected parasites harboring the *Pfmdr1* N86Y and Y184F alleles or the *Pfmdr1* haplotype in new infections emerging from the liver after clearance of artemether. Our study is in agreement with another work done at Uganda which reported significant increase in the prevalence of *Pfmdr1* N86Y and Y184F alleles from pretreatment samples to post treatment ($P > 0.0001$) (Dokomajilar *et al.*, 2006) Another report from Zanzibar (Sowunmi *et al.*, 2007) showed a significant accumulation of *Pfmdr1* N86 and Y184 alleles among patients who had parasites after AL treatment. There are some limitations inherent in this work and the results are analyzed as such. Our study did not differentiate recrudescence from reinfection by genotyping with the view of observing the strength of AL in protecting a patient for up to 28 days in an area of high endemicity and samples were not sequenced to confirm the presence of SNPs as reported. However, further studies are needed to obtain the sequences of the *Pfmdr1* gene in this parasite population

CONCLUSION

In conclusion, our study has demonstrated that there is high prevalence of *Pfmdr1* N86Y and F184Y and that the prevalence of mutated alleles of *Pfmdr1* is higher in patients who had artemisinin resistance than in those who had good response hence is strongly associated with artemisinin resistance in Enugu, Nigeria. It has also shown that presence of artemether-lumefantrine treatment induced genetic variation in the *Pfmdr1* gene.

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