Host candidate gene polymorphisms and associated clearance of *P. falciparum* amodiaquine and fansidar resistance mutants in children less than 5 years in Cameroon

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Background: In this *post-hoc* analysis, we determined the influence of single nucleotide polymorphisms in host candidate immune genes on the outcome of drug resistant malaria in Cameroon.

Methods: Human DNA from 760 patients from a previous clinical trial was subjected to mass spectrometry-based single nucleotide polymorphism (SNP) genotyping. Allele frequencies of candidate immune genes were calculated for 62 SNPs on 17 human chromosomes for their possible involvement in clearance of drug-resistant parasites with the triple mutations of *pfprt*76T, *pfmdr*86Y, and *pfmdr*1246Y (TY) and *pfdhfr*51I, *pfdhfr*59R, *pfdhfr*108N, and *pfdhps*437G (IRNG) which were determined by dotblot or PCR-restriction analysis. Differences in SNP frequencies and association analysis were carried out by comparing Chi-square odds ratios (ORs) and stratified by Mantel-Haenszel statistics. An adjusted *P* value (OR) <0.0008 was considered significant.

Results: Post-treatment drug failure rates were amodiaquine (36.4%); sulphadoxine/pyrimethamine-amodiaquine combination (15.4%); and sulphadoxine/pyrimethamine (18.1%). SNPs in IL22, IL-4R1, and CD36 appeared to have been associated with clearance of resistant parasites [*p* = 0.017, OR (C allele):1.44, 95% CI (OR): 1.06–1.95]; [*P* = 0.014, OR = 1.31, 95% CI (OR): 1.07–1.83]; [*P* = 5.78 × 10^{−5}, OR = 0.27, 95%CI (OR): 0.13–0.54], respectively, with high fever (>39°C for 48 hours) [IL-22, *P* = 0.01, OR = 1.5, 95% CI (OR): 1.8–2.1] and also in high frequency among the Fulani participants [*P* = 0.006, OR = 1.83, 95% CI (OR): 1.11–3.08]. The CD36-1264 null allele was completely absent in the northern population.

Conclusion: Independent association of SNPs in IL22 and IL-4 with clearance of amodiaquine- and sulphadoxine/pyrimethamine-resistant parasites did not reach statistical significance, but may suggest that not all drug-resistant mutants are adversely affected by the same immune-mediated mechanisms of clearance.

Keywords: Interleukin-22, Parasite clearance, Fever clearance, Drug resistance markers, Sulphadoxine/pyrimethamine, Amodiaquine, Immune response

Background

Malaria remains a public health threat in Africa where the therapeutic property some of the cheap, most available, and easily affordable drugs has been

compromised due to drug resistance.^{1,2} Today, the mainstay for case management in most settings is the use of an artemisinin-based combination. The rationale for the use of this combination relies on the rapid clearance of drug resistant parasites, reduction of transmission,³ protection of partner drug against resistance, and rapid fever reduction.^{4,5} The ability to clear *Plasmodium falciparum* parasites in humans

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depend on the drugs used for treatment and also on host immune response. Effector mechanisms parasites use for evading clearance in malaria involve genetic alterations in genes involved in drug metabolism,⁶ but also on genetic adaptations in drug targets. For *Plasmodium falciparum*, these processes are widely used both for modulation of host-parasite relationships for survival and propagation. Within the erythrocyte, *P. falciparum* parasites exist as haploid organisms. In this life cycle stage, point mutations in genes encoding transport molecules or drug metabolizing genes can be deleterious¹ due to lack of compensatory genetic mechanism. Hence, resistance to sulphadoxine/pyrimethamine (SP) is conferred by amino-acid substitutions in the dihydropteroate synthase (*dhps*) gene codon 436(S436A/F), 437(A437G), 540(K540E), 581(A581G), and 613(A613S/T) and the dihydrofolate synthase gene codons 51, 59, and 108 (N51I+C59R+S108N, respectively) in a stepwise fashion. A combination of mutations in both genes (haplotypes) confer moderate-to-high grade resistance to SP (Table 1). In east Africa, high grade resistance to SP is conferred by five mutations (N51I+C59R+S108N+A437G+L540E) in the *dhfr* and *dhps* genes, respectively. Similarly, mutations in the *Plasmodium falciparum* chloroquine transporter gene (*pfcr*) at codons 72–76 and at the *Plasmodium falciparum* multidrug transporter gene 1 (*pfmdr1*) at codons 86, 184, 1034, and 1246 confer resistance to chloroquine and amodiaquine, respectively, although evidence for resistance results principally from point

mutations at codons 76 (K76T) of *pfcr* and 86(N86Y) of *pfmdr1*, or a combination of both mutations.⁴³ The following table indicates some of the resistance conferring mutations of *Plasmodium falciparum* mutations and haplotypes tested in the present analysis.

The observations that antimalarial treatment efficacy depends not only on intrinsic drug activity, but also on age as well as on transmission intensity indicates additional host factors are involved in shaping the treatment outcome.^{7,8,12} Sulphadoxine–pyrimethamine clearance of malaria infections was shown to be enhanced in individuals heterozygous for the sickle cell trait (HbAS) compared to individuals with the normal hemoglobin form (HbAA).⁴² The authors found those with HBAS at reduced risk of treatment failure by day 7 post-drug treatment among all age groups and particularly in children below 6 months, an observation which could partially be explained by materno-fœtal transfer of anti-malarial antibodies during birth.⁴² Clearance of *falciparum* malaria is also known to be mediated by immune mechanisms with antibodies and cytokines.^{9,10} This is also the case for drug-resistant malaria.^{7,9,36} Genetic variations, notably single nucleotide polymorphism (SNPs) within genes encoding innate immune factors including cytokines, their receptors, as well as immunomodulatory molecules, are implicated in/resistance to malaria parasites.^{10,11,32} This may explain in part why some patients naturally clear infections while others do not. In a mouse model of malaria Mohan *et al.*, administered a combination of the pleiotropic cytokine

Table 1 Some genes, mutations, and haplotypes associated with resistance to amodiaquine and sulphadoxine/pyrimethamine treatment

Gene	Abbreviation	Codon	Mutation	Short form of mutation	Resistance haplotype	Phenotype
<i>Plasmodium falciparum</i> dihydrofolate reductase	<i>dhfr</i>	51	leucine to Isoleucine	L51I	IRN	Decreased susceptibility to pyrimethamine
		59	Cysteine to arginine	C59R		
		108	Serine to asparagine	S108N		
<i>Plasmodium falciparum</i> dihydropteroate synthase	<i>dhps</i>	437	Alanine to glycine	A437G	IRNG*	Decreased susceptibility to sulphadoxine
		540	Lysine to glutamic acid			
	<i>dhfr + dhps</i>	All above	All above			Sulphadoxine–pyrimethamine resistance conferring
<i>Plasmodium falciparum</i> chloroquine resistance transporter	<i>pfcr</i>	76	Lysine to threonine	K76T		
<i>Plasmodium falciparum</i> multidrug resistance transporter	<i>Pfmdr 1</i>	86	Asparagine to tyrosine	N86Y		
		184	Tyrosine to glutamic acid	Y184E		
		1246	Aspartic acid to tyrosine	D1246Y		
	<i>Pfcr + pfmdr1</i>	76 + 1246		K76T + N86Y	TY*	Amodiaquine resistance conferring

Note: *Haplotypes considered in the present analysis.

The TY resistance conferring haplotype was considered for association with amodiaquine resistance, while IRNG resistance conferring haplotype was considered for association with sulphadoxine–pyrimethamine resistance.

IL-12 and low dose chloroquine and demonstrated that addition of this cytokine promoted the IFN- γ -mediated Th1 effector responses and upregulation of erythropoiesis. This report showed that the combination also generated protective antimalarial antibody responses¹³ suggesting that immune responses to malaria can enhance the efficacy of anti-malarial drugs.³⁷ Therapeutic outcomes will, therefore, vary as a function of the level of immune activation. In a West Africa study on the susceptibility of malaria by distinct ethnicities, Modiano *et al.*^{13,32} found the Fulani to be less susceptible to malaria compared to their sympatric neighbors and associated this to a more efficient induction of innate response in the former. Of note, in variations in IL-4, an anti-inflammatory cytokine was shown to upregulate antimalarial specific IgE production in the Fulani of West Africa, thereby contributing to parasite clearance in this ethnic group.³³ Similar observations were made in a study in Burkina Faso where blood parasitemia levels were shown to be genetically linked to the 5q31–q33 chromosomal region,¹⁶ a hotspot closely linked with immune clearance of intracellular parasites. The Fulani group may be producing a more efficient response to malaria^{13,31} than their sympatric neighbors. We set out in this study to investigate natural variations in immune genes and associated clearance of parasites with drug resistance mutations in three major geographical locations and among the Fulani in northern Cameroon. The results reflects the result of a *post-hoc* analysis on samples obtained from a previous multi-center clinical trial assessing the clinical efficacy and safety of SP and amodiaquine (AQ) combination used in Cameroon as an interim measure for managing uncomplicated malaria when the recommended artemisinin-based combination therapies were not yet available to the public.

Methods

Study setting, parasite, and clinical drug responses

Clinical and parasitological outcomes among children less than 5 years were assessed in a randomized, controlled, and double-blinded clinical trial investigating the efficacy and safety of AQ plus SP in three ecological regions of Cameroon. This combination was used as an interim measure when artemisinin-based combinations were desired but not yet available in Cameroon. Outcomes were assessed according to the guidelines provided by the WHO¹⁷ as early treatment failure (ETF), late clinical failure (LCF), late parasitological failure (LPF), or adequate clinical and parasitological response (ACPR).¹⁸ Parasite DNA extracted from blood spots using the modified chelex-100 method as described¹⁹ was used to genotype of point mutations on the *pfprt*, *pfmdr-1*, *dhfr*, and *dhps* genes using sequence-specific oligonucleotide probes as described by Pearce *et al.*,²⁰ with tagged digoxigenin, tagged probe, and alkaline

phosphatases' ability to break down of a chromophore to observe enhanced chemluminescence. Recurrent infections were classified as recrudescing or reinfection based on comparing the genetic profiles of parasites before or after treatment. The genetic profiles were established from comparing gene fragment sizes of three known polymorphic antigen markers — merozoite surface protein 1, merozoite surface protein 2, and the glutamine-rich protein (*msp 1*, *msp 2*, and *glurp*, respectively) genes. If a pre-treatment isolate had similar *msp 1*, *msp2*, and *glurp* profiles like a recurrent parasite, it was classified recrudescence. Otherwise, it was a reinfection. This was necessary to determine true treatment failure.

SNP selection and assay design

The candidate gene approach was to genome scanning was employed primarily based on a growing body of information of immune markers that were known or suspected to be associated with susceptibility or resistance to malaria. Single nucleotide polymorphisms of immune genes were selected based on a desk review of known immune genes and markers which were demonstrated to influence susceptibility or resistance to infectious diseases, notably malaria. Most of the markers described were found in the SNP database hosted by the National Centre for Biomedical Information (<http://www.ncbi.nlm.nih.gov/projects/SNP/>, 2009 Genome build). These candidate genes were principally cytokines, chemokines, lymphokines, or their receptors. The criteria used for selection included marker spacing, known functional attributes of the SNP, the minor allele frequency, and the possibility of designing genotyping assays in the Sequenom's Mass Array Platform (<http://www.sequenom.com/iplex>). Assay design using Spectro DESIGNER allowed for up to six SNPs to be interrogated in a single well.

DNA extraction and genotyping

Human DNA samples extracted using chelex-100 were purified and quantified using the Picogreen quantification kit²¹ and subjected to primer extension pre-amplification as described²² before mass spectrometry-based genotyping. For PCR amplification, the DNA samples were subjected to denaturation at 94°C for 5 minutes, and followed by 40 cycles of amplification at 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 30 seconds per cycle; and a final extension of 72°C for 3 minutes. Extend products were subjected to the following primer extension conditions before mass spectrometry: briefly, an initial denaturation at 94°C for 30 seconds, denaturation consisting of 40 cycles at 94°C for 5 seconds each, annealing and extension at 52 and 80°C for 5 seconds respectively for a total of five cycles and followed by a final extension at 72°C for 3 minutes. PCR products were

cleaned for downstream reactions by removal of unincorporated dNTPs from amplified products. This was carried out by incubating the amplified products with shrimp alkaline phosphatase (SAP) as follows. Two microliters of amplified cocktail was added to 6 μ l of SAP mastermix containing 120 μ l of $\times 10$ SAP buffer and 218 μ l of 1.7 U SAP and the mixture incubated at 37°C for 40 minutes and then at 80°C for a further 10 minutes. Several layers of quality control were applied in the entire genotyping process. These included checks for primer–primer, primer–product, or product–product interactions to minimize non-specific PCR extensions. Primer extension products were desalted, and cleaned with 6 mg of resin each in 16 μ l of nuclease free water. The extension products were then spotted on 384-well SpectroCHIPS using a nanoliter sample dispenser and analyses selected SNPs through matrix assisted light desorption ionization time-of-flight mass spectrometry using a compact Sequenom mass spectrometer (Sequenom Inc., San Diego, CA, USA). The extension products ionize in an electrostatic field aided by the expanding matrix itself receiving energy from a laser source. Extension product sizes are separated and accelerated towards a detector in this electrostatic field based on their mass/charge ratio. In this procedure, genotype calls are made in real time. The assay results consisting of spectral data were initially evaluated according to the fraction of assay successes per plate. An assay failed if it did not meet minimal criteria of mass spectral quality as determined in real-time by the Mass ARRAY software. A threshold of 10% was imposed as the limit of success for plate quality control.

Statistical analysis

Markers of drug resistance were identified and the prevalence of each marker determined for each study site. The Chi-Square test was used to compare response to therapy between different treatment arms and ecology for all categorical variables of the therapeutic outcome (ETF, LCF, LTF, ACPR). For the purpose of this study, parasite clearance was defined as the disappearance of pre-treatment parasites without subsequent recurrence, irrespective of whether recurrence is a reinfection or a recrudescing parasite. Patients that experienced LCF, ETF, and LPF were classified as failures, while patients that experienced ACPR were classified as successes. The baseline frequency of resistance conferring mutations was compared between those who failed treatment and those who experienced ACPR to test for marker selection or associations with treatment outcome using Fisher's exact test. For all participants, candidate SNP genotyping was evaluated by estimating different SNP frequencies through counting. The

difference in distribution of SNPs between populations failing or succeeding to clear AQ or SP-resistant mutants (*pfprt*-76 and *pfmdr1*-86 for AQ resistance and *dhfr*-51, 59, and 108 and *dhps*-437 for SP) was assessed by the use of Chi square test. Association between SNPs frequencies and treatment response was analyzed by performing pairwise comparisons of odds ratio (OR) for different host candidate alleles and treatment failure or success. The Mantel–Haenszel statistic was applied to adjust for the potential confounding factor of ethnicity in the general analysis. Furthermore, to evaluate if patients from the north may be using the same immune strategy to adequately respond to AQ resistant mutants, the distribution of candidate gene SNPs among patients who experienced ACPR with AQ related mutants in the northern region and southern region was compared using Fisher's exact test. Finally, the distribution of candidate gene SNPs among the Fulani participants clearing AQ resistant parasites was performed. Another subgroup analysis was further performed to assess the effect of high and persistent pre-treatment temperature on clearance of parasites. In this analysis, the association between clearance status and pre-treatment temperature was done using the Kruskal–Wallis test. Pre-treatment temperature was high, defined as axillary temperature at enrolment $>39^{\circ}\text{C}$ and persistent, defined as high temperature for >48 hours post-treatment. We considered adjusting for multiple SNP testing in our analysis. The probability of a type I error is generally set at 5%. In a set of n tests, the overall risk of a type I error becomes higher than 5% defined by an upper bound according to the conservative Bonferroni's inequality. Overall- $\alpha < n \times \alpha'$ where α' is the type 1 error for each of n tests set at 5%. Therefore the adjusted alpha level for each SNP analysis after Bonferroni correction is given by α'/n , in order to keep the overall alpha level at 5%.⁴⁴ An adjusted P value of 0.0008 (0.05/62) was therefore considered as the significance threshold for all SNP association testing. The Hardy–Weinberg test²³ was performed for all markers to evaluate if genotype/allele frequencies were consistent with expected frequencies under Hardy–Weinberg Equilibrium (HWE). Variants whose allele frequencies deviated from HWE were eliminated from subsequent analysis. See supplementary material online for this article. (www.maneyonline.com/DOI/suppl/10.1179/2047773214Y.0000000159).

Ethics review

Ethical approval for the study was obtained from the Institution Review Board of the Cameroon Baptist Convention Health Board, the National Ethics Committee of Cameroon and from the Ethics Review

Figure 1 Proportion of amodiaquine resistance conferring alleles in the three study sites. This figure indicates that except for Garoua in the north of Cameroon, the prevalence of 654 AQ resistance conferring alleles was high and comparable in Mutengene and Yaounde.

Committee of the London School of Hygiene and Tropical Medicine. The trial objectives and procedures were interpreted in French, English, or a relevant local language to the parents or guardians of each potentially eligible study participant and answers to any questions were given. Informed consent was obtained from the parents/guardians by signature or thumb mark. The trial was registered in the NIH clinical trials database (NCT00146718).

Results

Drug resistant mutations and efficacy of AQ and SP

The efficacy and safety of the combinations assessed in this study is reported elsewhere.¹⁸ Table 2 summarizes the efficacy findings of this clinical trial by site and by drug arm.

The prevalence of the mutations studied was recorded in the different study towns with overall significantly lower prevalence of all mutations in Garoua compared to Yaoundé and Mutengene ($P = 0.0001$). The *pfprt*76T for instance had a prevalence

of 76.8% in Yaoundé, 87.1% in Mutengene, and 31.7% in Garoua, while that for the *pfmdr1* 86Y was respectively 76.1%, 73.8%, and 22.1% (Fig. 1)

The differences in the frequencies of the mutations studied by day 14 and day 28 post-treatment were minimal for *dhfr* ($P > 0.05$) but significant ($P = 0.017$) for *dhps* SGK in Mutengene (Fig. 2), indicating that parasites with *dhfr* mutations were cleared at a similar rate in response to SP drug treatment, but parasites with the *dhps* SGK mutation in Mutengene were not. Haplotypes constructed from different mutations that confer resistance to SP [*dhfr* codons 51I, 59R, and 108N and *dhps* codons 437G (IRNG)] and AQ [*pfprt* 76T and *pfmdr1* codons 86Y (TY)] are shown in Fig. 3. The combined frequency of drug resistant haplotypes in the *dhfr*, *dhps*, *pfprt*, and *pfmdr1* genes for all sites shows that AQ and SP resistance conferring haplotypes were high with the IRNG haplotypes being more prevalent than TY haplotypes in the parasite population. Distribution of *pfmdr1* resistance conferring mutant (*pfmdr1*-1246Y)

Table 2 Treatment outcome by drug arm and study site

Variable	Garoua (240)			Mutengene (260)			Yaounde (260)			Total
Number recruited	n=80	n=80	n=80	n=87	n=86	n=87	n=86	n=86	n=88	760
Drug	AQ	AQSP	SP	AQ	AQSP	SP	AQ	AQSP	SP	
ETF	10	3	10	3	4	14	1	0	8	53
LCF	1	2	5	7	2	7	4	2	12	42
LPF	8	6	7	6	6	11	8	10	12	116
Crude cure rates (%)	72	81.7	71	88.3	82.5	75.3	86.3	82.3	88	
PCR corrected cure rates (%)	71.2	80.9	70.1	79.2	81.9	62.5	80.3	76.2	67.5	
Lost to follow up	11	6	9	5	3	10	9	6	2	61

Note: AQ: amodiaquine, AQSP: amodiaquine/sulphadoxine/pyrimethamine combination, SP: sulphadoxine/pyrimethamine. ETF: early clinical failure, LCF: late clinical failure, LPF: late parasitological failure (source: Mbacham *et al.*, 2010).¹⁸

Figure 2 Prevalence of sulphadoxine/pyrimethamine alleles in the three study sites. This figure indicates that compared to Garoua, SP resistance conferring alleles were higher in Mutengene and Yaounde. The K540E mutation was found in one parasite isolate in Mutengene.

was not different among the sites and occurred only among very few parasite isolates at each site.

Association tests between different AQ resistance conferring haplotypes did not indicate any significant relationship with treatment response to amodiaquine. Table 3 above shows that both wild type and resistance conferring mutants of *pfmdr1* and *pfcr1* genes were proportionately distributed among those who succeeded or failed to clear parasites.

The CIRN/SGK haplotype, associated with resistance in our population was proportionately distributed among SP failures and those who adequately cleared infections. Association tests showed that none of the haplotypes were significantly associated with SP treatment response. The SGK haplotype tended to be associated with SP treatment failure but did not reach significant levels (Table 4). Only one sample

contained the SGE haplotype, which is known to confer high grade SP resistance in East Africa. This sample was obtained from circulating parasites in Mutengene where resistance conferring mutants to chloroquine were first described in Cameroon.

Meanwhile, there was no significant association between resistance conferring alleles and treatment response in all sites combined; the results showed most patients clearing both AQ and SP resistance conferring mutants in all three sites. This result indicates other factors, notably protective immune responses, may be associated with the clearance of mutant parasites at all three sites. It should be noted also that the proportion of some of these resistance conferring mutant parasites was a significant percentage of the total parasite population in the infections.

Allele frequencies of candidate immune genes and association with treatment outcome

Genotype call rate was high (>95%) among samples submitted for sequencing. As demonstrated by HWE tests, there were significant deviations for six SNPs in the population, showing that for more than 90% of the SNPs, the study population was fairly under HWE. Patients classified as failures included those cumulatively classified as early, late clinical, and late parasitological failures, while those classified as success were those who responded adequately both clinically and parasitologically and were seen, examined and formally terminated the study by day 28 post-treatment. Of the SNPs showing a significant relationship with clearance of the TY (AQ resistance conferring haplotype) (Fig. 1), the IL-22 was found to correlate with clearance resistant AQ parasites with a Chi-squared *P* value of 0.017 and an OR of the

Figure 3 Proportion of patients with SP and AQ resistance conferring mutations in the study population. This figure shows that for all study participants, a majority ($\geq 65\%$) of pre-treatment parasites carried resistant conferring alleles for both trial medications.

C allele of 1.44 [95% CI (OR): 1.06–1.95; $P < 0.05$] (Table 5a).

The IL-22 SNP was also found to be significantly more frequent among the Fulani study participants compared to the non-Fulani participants (Table 6), suggesting that IL-22 may be involved in mediating enhanced parasites clearance in this ethnicity. However, other SNPs, also found to be over-represented in the Fulani in this study included IL-10, IL-17, CC6, and TNF-SM2, indicating that the effect of IL-22 may be within a system of mediators associated with inflammatory pathway. On the other hand, analysis performed using the IRNG haplotype that confers resistance to SP showed that a SNP in the IL-4R gene (rs1805015) significantly correlated with clearance of IRNG containing parasites (OR for 'A' allele = 1.31, 95% CI = 1.07–1.63) with a P value < 0.05 (Table 5b). However, when the IL-22 SNP was

analyzed, it failed to reach statistical significance for clearance of SP-resistant parasites, suggesting that enhanced clearance of drug-resistant malaria parasites may occur by different immune mediators. On the other hand, an SNP in the IL-4 receptor (rs1805015) was significantly associated with clearance of parasites with SP resistance conferring mutations.

Polymorphisms were also analyzed to evaluate their associations with pre-treatment temperature (Table 6). Among patients with persistent day 0 temperatures greater than 39.0°C for 48 hours, analysis showed that the IL-22 SNP (rs2227491) significantly correlated with parasite clearance (OR = 1.5, 95% CI: 1.8–2.01). This analysis indicates that among drug-treated children with high and persistent pre-treatment temperature, children with IL-22 SNP (rs2227491) could have more circulating

Table 3 Association between wild and resistance conferring mutant alleles/haplotypes and amodiaquine treatment response in the AQ treatment cohort

Genes	Genotype status	Allele/haplotype	AQ treatment response				
			Failure	Success	Chi-square value	P value	OR (CI)
<i>Pfcr</i>	Mutant	<i>Pfcr</i> 76T	66	241	0.035	0.85	0.94 (0.46–1.88)
	Wild	<i>Pfcr</i> K76	12	41			
<i>Pfmdr1</i>	Wild	<i>Pfmdr1</i> N86	11	67	3.74	0.03	0.50 (0.25–1.02)
	Mutant	<i>Pfmdr1</i> 86Y	66	209			
	Mutant	<i>Pfmdr1</i> 184F	47	162	0.004	0.94	0.96 (0.36–2.54)
	Wild	<i>Pfmdr1</i> Y184	6	20			
	Wild	<i>Pfmdr1</i> 1246D	69	268	6.90	0.75	
<i>Pfmdr1 + pfcr</i>	Mutant	<i>Pfmdr1</i> 1246Y	7	7			
	Mutant	<i>pfcr</i> 76T + <i>Pfmdr1</i> 86Y	60	214	0.24	0.0.62	1.23 (0.52–2.93)
	Mixed	<i>Pfcr</i> 76T N86 + <i>Pfmdr1</i>	13	47			
	Wild	<i>Pfcr</i> 76K + <i>Pfmdr1</i> N86	7	31	0.13	0.72	1.16 (0.51–2.59)
	Mutant	<i>Pfmdr1</i> 76T + 86Y + 1246Y	10	23			
	Mixed	<i>Pfmdr1</i> 76T + 86Y + 1246D	63	268			
	Wild	<i>Pfmdr1</i> 76K + 86N + 1246D	8	37			

Note: Treatment outcome classification was based on the WHO 2003 Drug Efficacy Testing protocol. The results were corrected by using the polymorphic gene marker merozoite surface protein 2 (msp-2) to distinguish recrudescence infections from re-infections. Infections that contained but wild and mutant alleles of both genes were treated as mutants of both genes. *pfcr*: plasmodium falciparum chloroquine resistant transporter gene; *pfmdr-1*: plasmodium falciparum multidrug resistance gene 1; TY is the resistant haplotype of the combined *pfcr* and *pfmdr-1*; Chi-square: Chi-square, OR: odds ratio, CI: confidence interval; P value: significance threshold=0.05.

*Significant.

Table 4 Association between wild and resistance conferring mutant alleles/haplotypes and sulphadoxine/pyrimethamine treatment response in the SP treatment cohort

Genes	Genotype Status	Haplotypes	SP treatment response				
			Failure	Success	Chi-square value	P value	OR (95% CI)
<i>Dhps</i>	Wild	AAK + SAK	18	47	0.4	0.52	0.81 (0.44–1.51)
	Mutant	SGK + SGE + AGK	59	188			
<i>Dhfr</i>	Wild	CNCS	7	18	0.18	0.67	0.82 (0.32–2.04)
	Mutant	CIRN + CNRN + C1CN	74	232			
<i>Dhfr + Dhps</i>	Mutant	CIRN/SGK	75	201	0.11	0.74	0.83 (0.28–2.45)
	Mixed	CIRN/SAK	18	45			
	Wild	CNCS/SAK	5	11			

Note: Treatment outcome classification was based on the WHO 2003 Drug Efficacy Testing protocol. The results were corrected by using the polymorphic marker merozoite surface protein 2 to distinguish recrudescence infections from reinfections. Infections that contained but wild and mutant alleles were treated as mutants. *dhfr*: plasmodium falciparum dihydrofolate reductase gene; *dhps*: plasmodium falciparum dihydropteroate synthase gene; SGK, IRN are resistant haplotypes of the *dhps* and *dhfr* genes, respectively. Chi-square: Mantel-Haenszel Chi-square; OR: odds ratio, CI: confidence interval; P value: significance threshold=0.05.

drug-resistant parasites compared to children who did not carry the mutation.

There was a significantly lower prevalence of the CD36-T1264G heterozygote genotype (7% versus 18.4%) among participants having circulating forms of this mutant in the north compared to participants in the south. We estimated CD36 deficiency defined by the homozygous recessive (TT) condition at position 1264 of the CD36 gene. We observed a low prevalence of CD36 deficiency (<7%) in the general study population. On the other hand, when the distribution of the SNP was stratified by geographical location of study participants, we observe that CD36 deficiency (the GG homozygous condition of the CD36 gene) was completely absent among all the 120 participants clearing resistance conferring *P. falciparum* infections in the northern study population; and at low frequency in the southern population (4%). Overall, although significant associations were observed between the presence of some candidate immune gene SNPs and clearance of resistance conferring mutations and pre-treatment temperature, all except the CD36T1264G mutation did not demonstrate independent significance after testing

for multiple comparisons. The CD36T1264G mutation, on the other hand, significantly clustered in the population of participants that cleared parasites with AQ resistance mutation in the southern part of Cameroon.

Discussion

Our study reports varying prevalence of drug resistance mutations within three geographically distinct zones of Cameroon for AQ, SP, and AQSP. The proportion of resistance mutations was compared with rates of treatment failure to each drug. We found that no clear association existed between levels of treatment failure and resistance mutations, except for Mutengene where there was a relationship between parasite clearance and the *dhps*-437 mutants. In addition, we observed that levels of drug resistance mutations differed with study sites, with Garoua (North) being the site that has the least frequency of circulating mutant parasites. There was no significant risk of failure observed in infected patients that carried the *pfmdr1* alleles associated with diminished AQ response in line with published information.³⁸ The continuous mapping of the patterns of drug

Table 5a Association between parasite clearance and an interleukin-22 (IL-22) polymorphism

SNP (rs2227491)	Allele A (T)	Allele B (C)
Success (ACPR)	260	212
Failure (ETF + LCF + LPF)	184	104
Total	444	316
Analysis: IL-22/AQ	Chi-squared <i>P</i> value	0.017
OR for allele A	1.4419	
95% CI for OR	1.0674–1.9496	
<i>P</i> value for OR	<0.05	

Note: ETF: early treatment failure; LCF: late clinical failure; LPF: late parasitological failure; ACPR: adequate clinical and parasitological response. All classifications based on WHO 2003 protocol for clinical efficacy of antimalarial drugs. The analysis involved correlation between children failing treatment with resistance conferring mutations in the *pfcr* and *pfmdr1* mutations (Fig. 1 above) and SNPs in the immune genes versus children who successfully cleared their parasites with these mutations.

Table 5b Association between parasite clearance and an interleukin-4 receptor (IL-R) polymorphism

SNP (rs1805015)	Allele A(T)	Allele B (C)
Success (ACPR)	136	212
Failures (ETF + LCF + LPF)	68	64
Total	204	276
Analysis (IL-4R)	Chi-square <i>P</i> value	0.014
Odds ratio for Allele A	1.31	
95% CI for OR	1.07–1.63	
<i>P</i> value for OR	<0.05	

Note: ETF: early treatment failure; LCF: late clinical failure; LPF: late parasitological failure; ACPR: adequate clinical and parasitological response. All classifications based on WHO 2003 protocol for clinical efficacy of antimalarial drugs. The analysis involved correlation between children failing treatment with resistance conferring mutations in the *dhfr* and *dhps* genes (Fig. 3) and SNPs in the immune genes versus children who successfully cleared their parasites with these mutations.

Table 6 Analysis of association between mutant SNPs with ethnic group (Fulani), SP, AQ, and pre-treatment temperature

SNP	Parameter	Gene	Chi-square <i>P</i> value	Odds ratio	95% CI for OR	Adjusted significance
rs3024500	Fulani	IL-10	0.002	0.594	0.43–0.81	NS
rs6780995		IL-17	0.002	0.595	0.43–0.81	NS
rs1801033		C 6	0.013	0.679	0.51–0.90	NS
rs1799964		TNF-SM2	0.003	0.286	0.11–0.69	NS
rs2227491		IL-22	0.006	1.830	1.11–3.03	NS
rs1805015	SP	IL-4R	0.014	1.311	1.07–1.63	NS
rs2227491	T°C (>39°C)	IL-22	0.013	1.502	1.80–2.01	NS
rs2227491	AQ	IL-22	0.017	1.440	1.07–1.94	NS

Note: rs numbers correspond to SNP reference in the dbSNP database hosted by the NCBI. The SNP database used was the August 2009 build (<http://www.ncbi.nlm.nih.gov/projects/SNP/>). AQ: amodiaquine, SP: suphadoxine and pyrimethamine, T°C: temperature. Correlation between frequency of SNP and clearance of parasites with resistance conferring mutations was stratified according to high and persistent day 0 temperature (>39°C for 48 hours) and tested using the Mantel-Haenszel statistics. NS: not significant. Adjusted significance was based on re-assessment of the association analysis taking into consideration the corrected *P* value obtained from the Bonferroni conservative approach as described in the section on 'Statistical analysis'.

resistance to antimalarials can be performed using data gained by fast and efficient molecular methods and can be used to support policy changes.²⁴ Our analysis of infecting parasite genotypes and treatment outcome did not show any strong and independent association between known parasite resistance markers and treatment response.

The host immune response represents a key factor that may interfere in the relationship between levels of drug resistant markers and observed treatment failure^{35,36} in *in vivo* drug studies. In individuals exposed to malaria for their first time, infection with the malaria parasite does not always lead to severe disease. Rather, a wide range of outcomes are possible ranging from death to resistance to infection. This range of responses evokes different possible innate immune mechanisms of protection.^{15,25} These mechanisms are often mediated through genetic polymorphisms in molecules involved in the pathogenesis of malaria. Hence, in some patients on antimalarial treatment, observed variations in treatment responses could be the outcome of variation in immune genes involved in malaria pathogenesis. To further assess the role of these variations in the outcome of treatment, we also investigated the association in SNP variation among patients that either cleared or failed to clear their parasites and the prevalence of parasite markers though to be involved in resistance to AQ/SP. The results show a number of cytokines to be involved in parasite clearance. Among the tested SNPs, only one SNP in IL-22 (*rs2227491*) showed a significant association with parasite clearance among the Fulani. When compared in D0 high fever producers, this association was significant for the same SNP in IL-22. These results suggest that this cytokine might be involved in a host response network related to parasite clearance and fever resolution. To our knowledge, this is the first report of the association between treatment response and SNPs in immune mediators in Cameroon's multi-ethnic population and pointing to an interesting perspective of the development of protective immune responses to malaria. The *rs2227491* SNP is an IL22 mutant allele resulting from a switch from the ancestral T to the derived mutation C change upstream of exon 5 of the IL-22 gene. IL-22 is a pro-inflammatory immune regulatory cytokine located on Chr 12, which is also related to IL10 and mediating Th17 function.²⁶ This cytokine is abundantly produced by cells of the innate immune system including gammadelta-T cells, natural killer cells, Th17 cells, and LT α i-like cells and Th22 cells. IL-22 is capable of inducing inflammatory mediators such as serum amyloid A protein, alpha-1 antitrypsin, and haptoglobin. IL-22 shares the IL-10RB, receptor chain with IL-10, but requires its own specific

receptor chain (IL-22R) for signal transduction.²⁷ The involvement of this cytokine in the modulation of the acute phase reactants suggests that in malaria, this cytokine may mediate early pro-inflammatory responses critical for parasite clearance. In a recent study investigating the differential expression of gene and protein products in peripheral blood mononuclear cells among HIV discordant couples and long lasting resistance to HIV-1, Missé *et al.* observed an over production of several proteins involved in innate response including IL-22 and a group of peroxiredoxins.²⁸ They suggested a role for the involvement of IL-22 in resistance to HIV-1, including its effect in the production of serum amyloid A, a formyl peptide receptor agonist that modulates CCR5 expression and hence, HIV entry.

Similarly, in a large case-control study in the Gambia, SNPs in *IL-22* gene were observed to be associated with susceptibility or resistance to severe malaria. A protective role for pro-inflammatory cytokines IL-17 and IL-22, via TH1 response inhibition, has been observed in a study of individuals co-infected with malaria and HIV.²⁹ In our study, the observed IL22 SNP may result in an increase in the expression of this protein in the liver resulting in hepatoprotective effects. In addition, a more efficient mechanism of clearance via modulation of acute phase reactants by IL-22 may be possible. Another possibility could be that IL-22 may simply be a marker that exists in linkage disequilibrium with other neighboring SNPs or genes. The IL-22 SNPs occur within 100 kb of the interferon gamma gene, although only weak linkage disequilibrium association was observed in one study for the genes in question.³⁰ More recently, Xie *et al.* showed that in *Plasmodium chabaudi* infection, IL-22 produced in the liver by CD8⁺ T cells contributed to protection from lethality from parasitaemia.³¹ Indeed, the authors found that lack of IL-22 resulted in 50% mortality among mice within 12 days of infection and weight loss at the peak of infection. The observations whereby IL-22 contributes to clearance of drug resistant malaria parasites is akin to observations of immune responses observed among the Fulani and the Dogon in Mali, two ethnic groups living in sympatry. An analysis of the innate immune responses in children from these two ethnicities has shown an inhibition of dendritic cell subsets, suppression of Toll-like receptor-stimulated cytokine responses, decrease in the frequency, and activation of circulatory monocytes as well as a decrease in TNF α /IL10 ratio. Following these observations, it seems likely that parasites induce suppression of the antigen presenting cell function in the non-Fulani children, resulting in a deficient and delayed activation of the acquired immune responses.³² This in turn influences early clearance of

parasites. However, the weak association between clearance of drug resistant infections and the immune mediators observed in our study did not support this observation.

The more efficient response among the Fulani could be maintained by upregulation of IL-4R on CD8⁺ cells, which has been shown to be a requirement for the development of protective memory against liver stage parasites.³⁴ The IL-4R SNP *rs1805015* is a missense mutation (T→C) resulting to a serine to proline change at position 503 of the interferon regulatory factor 1 protein. This mutation may be functionally implicated in the increased expression of interferon gamma. The IL-4R also binds to IL-13, which may contribute to its many overlapping functions with IL-13. IL-4, together with IL-13, IL-3, IL-5, and IRF-1, forms a cytokine gene cluster in the 5q31–q33 chromosomal region shown to be strongly associated with the interferon gamma mediated control of blood parasite density.^{16,33} Although SNPs in IRF-1 and IL-13 signals were obtained from our studies, their association with parasite clearance was equally weak.

Individuals heterozygous for 1264G were more likely to harbor parasites with AQ-sensitive alleles compared to those without the SNP. This difference may suggest that this SNP may be a more important determinant in the rate of clearance of AQ-resistant parasites in the Southern population compared to the Northern population. Furthermore, the absence of CD36 deficiency in the Northern ecology lends support to the proposition that the heterozygote mutant SNP may be associated with a faster rate of clearance of AQ-resistant parasites in the southern population in Cameroon. In a pooled analysis by Fry *et al.*,³⁹ no association was found between 1264G heterozygote and susceptibility to severe malaria, although a heterozygous allele was suspected to be associated to severe malaria susceptibility in the Gambia.⁴⁰ In a similar attempt to define the role of the mutation in children,⁴¹ malaria-infected children were followed up for a period of 1 year and the outcome of, and infection status after exposure to GPI antigens based on CD36 1264 allelic types were evaluated. They found that CD36-deficient children (CD361264 TT-homozygous recessive) failed to produce protective anti-GPI antibodies and were more susceptible to infection in the subsequent transmission season compared to children without the deficiency genotype. Although the frequency of the CD36 1264 null allele was small (4%) in the southern non-Fulani-dominated study participants clearing resistant infections, it was completely absent in the northern Fulani-dominated study participants, suggesting that mechanisms of clearance may occur differently or be related to study participant genetic background.

Study Limitations

Our study should be interpreted with some caution due to limited sample size for association analysis. It should be noted that the sample size was determined to test for clinical efficacy and safety and not for association analysis, which would have been much larger. In addition, the contribution of minor alleles in defining treatment outcomes would be possible only with very large samples size and population sub-structuring as was observed in a hospital-based and population-based study³⁵ evaluating candidate malaria susceptibility/resistant genes. Nevertheless, these findings flag the involvement of IL-22, IL-4R1, IL-10, IL-17, CD36, and TNF-SM2 in protective responses to malaria infections and treatment in Cameroonian children. This preliminary observation also points to the necessity of further studies to elucidate the role of natural immunity in mediating antimalarial treatment outcomes in different populations.

Disclaimer Statements

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Conflicts of interest All authors declare no conflict of interest.

Ethics approval Ethical approval for the study was obtained from the Institution Review Board of the Cameroon Baptist Convention Health Board, the National Ethics Committee of Cameroon and from the Ethics Review Committee of the London School of Hygiene & Tropical Medicine. The trial was registered in the NIH clinical trials database (NCT00146718).

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