

**Polymorphisms in *Plasmodium falciparum dhfr*
and *dhps* genes and age related *in vivo*
sulfadoxine-pyrimethamine resistance in malaria-
infected patients from Nigeria.**



BY

Tambo Ernest

Malaria Research Laboratories,

Institute for Advanced medical Research and Training,

College of Medicine

UNIVERSITY OF IBADAN IBADAN,

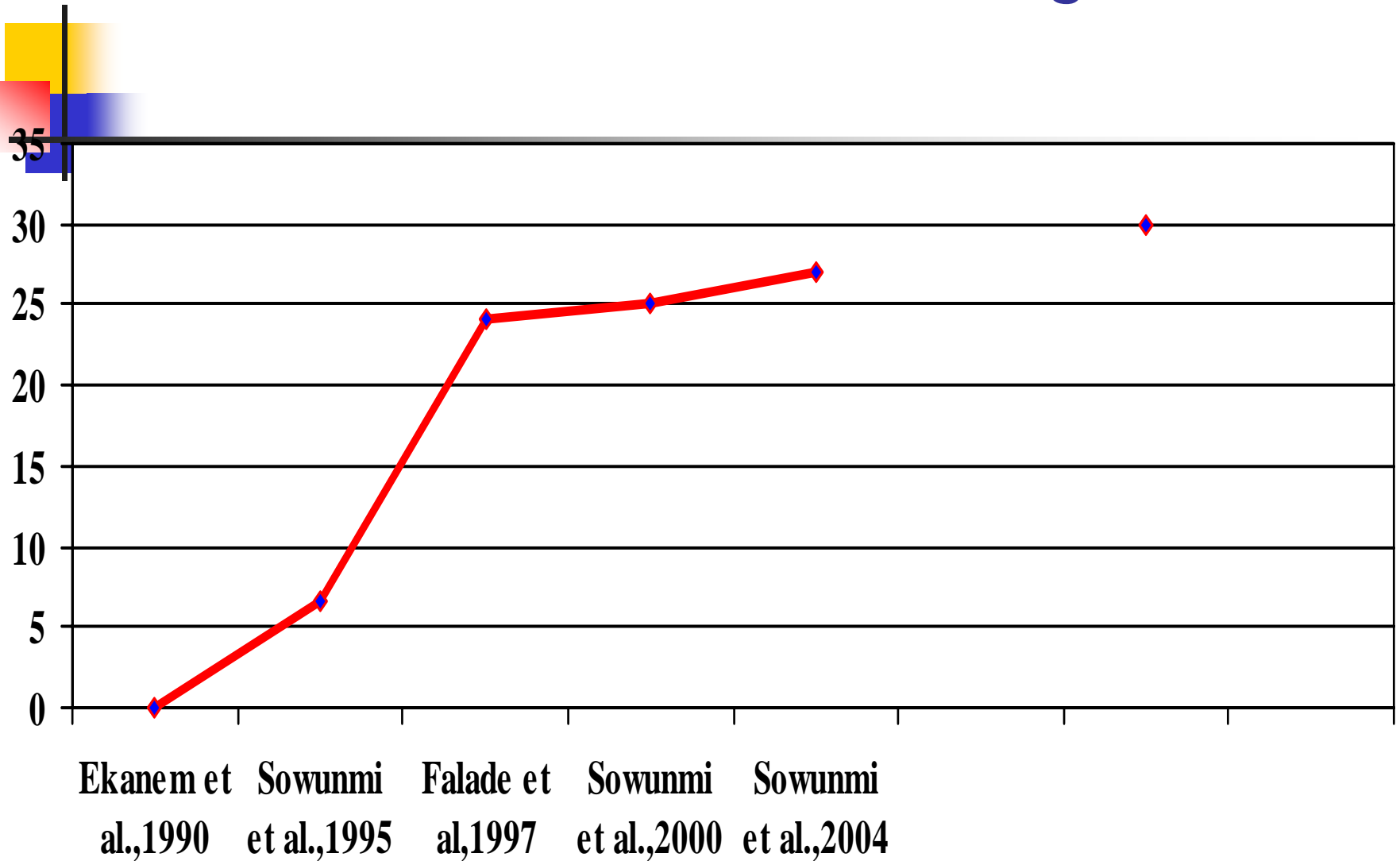
NIGERIA



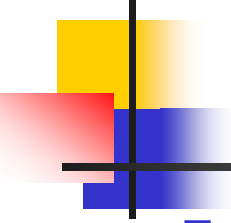
THE BURDEN OF *PLASMODIUM FALCIPARUM* RESISTANCE TO ANTIMALARIAL DRUGS

- Resistance of *P.falciparum* to currently available antimalarial drugs including CQ and S-P confounds efforts to control malaria in disease endemic countries.
- Increased morbidity and mortality has been documented especially amongst the risk groups.
 - Children under the age of 5 years ,
 - Pregnant women and
 - Non-immune travelers.

Epidemiology *Plasmodium falciparum* resistance to SP in Nigeria



CAUSES OF SP RESISTANCE

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- Resistance to SP is caused by accumulation of point mutations in the dihydrofolate reductase (*dhfr*) and dihydropteroate synthase (*dhps*) genes, due to
 - * Progressive selection as result of drug pressure.
 - * Increasing self-medication of antimalarial drugs in rural semi-urban and urban communities.



AIM OF THE STUDY

- **A long-standing goal in malaria research has been to use molecular predictors or markers of drug resistance as a rapid means of surveillance to promote evidence-based antimalarial drug treatment policy formulation.**



What is still unknown?

- Despite the understanding of the molecular mechanisms of resistance of *P.falciparum* to antifolate drugs, the determinants of treatment failure are still unclear in malaria settings in Africa.
- Moreover, the intrinsic parasite resistance is still a major limitation determining treatment outcome and remains a challenge for scientists.



SPECIFIC OBJECTIVES OF THE STUDY

- * Determine mutations or sets of polymorphisms in *P.falciparum dhfr* and *dhps* genes that could independently predict treatment failure.
- * Investigate the relationship between the parasite genotype, host immunity and response to SP treatment.
- * To establish a simple and reliable markers to predict SP treatment failure in different age groups in Nigerian children.

Materials and Methodology



■ Study Site and population:

- The study was carried out at the Malaria Clinic, Institute for Advanced Medical research and training of the MRL, University College Hospital , Ibadan, Nigeria from April 2003 to December 2004.
- The protocol for the study was approved by the Joint UI/UCH Institutional Review Committee and the Harvard School of Public Health Human Subjects committee(HCS).
- 118 patients aged 6 months-12 years with signs and symptoms of acute uncomplicated falciparum malaria were enrolled and treated with Sulfadoxine-Pyrimethamine(SP).



Patient's treatment and follow-up

- *** Patients were treated with standard single oral doses of sulfadoxine-pyrimethamine(SP) corresponding to 25mg/kg of body weight sulfadoxine component)**
- *** Follow-up with clinical and parasitological evaluations was done daily for seven days and then on day D14, D21 and Day 28.**
- *** Treatment outcomes were based on WHO standard protocol,(1996).**



Identification of mutations in *P. falciparum* *dhfr* and *dhps* genes

- Parasite genomic DNA was extracted from blood samples on dried filter paper using chelex extraction method.
- Five microliters of extracted DNA was added into 25µl final reaction mixed for primary amplification (Nested I) of specific region of interest in *P.falciparum dhfr* and *dhps*.
- One microliter of Nested I amplicon was used for Secondary amplification(Nested II) and then subjected to restriction-fragment-length-polymorphisms(RFLP) analysis.
- Primers and amplification conditions for the *dhfr* and *dhps* were based on those described by Duraisingh *et al.*,(1998).



PCR determination of *p.falciparum* population in malaria infected-patients

- **MSP-2 characterization was performed on all paired pre- and post-treatment parasites samples obtained from patients and analyzed to distinguish true treatment failures or recrudescence from new or re- infection.**
- **The complexity of infection was calculated as the average of number of distinct fragments of FC27 and IC1/3D7 per PCR-positive sample(Happi *et al.*, 2004).**

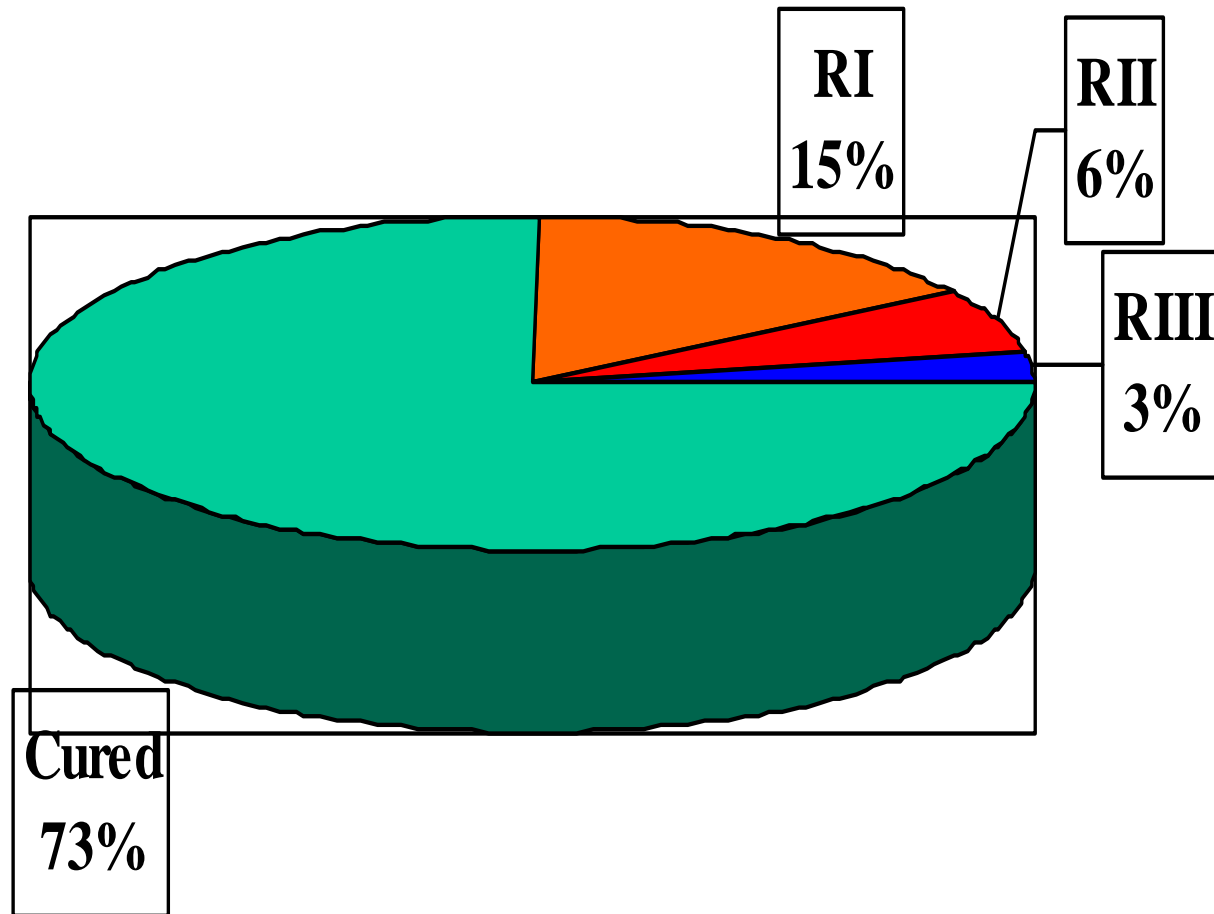


Results

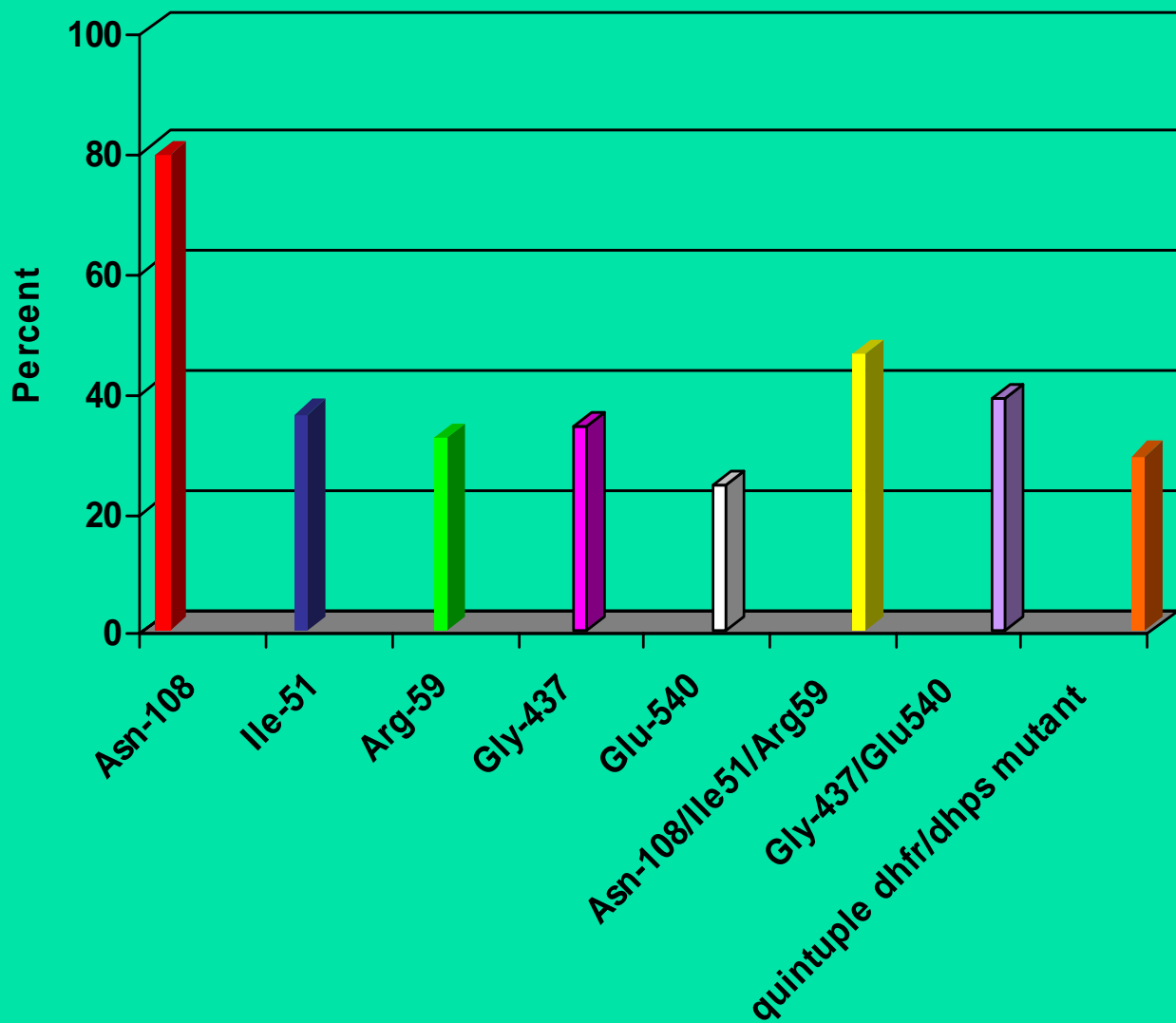
Demographic and clinical characteristics of patients at enrollment

No. of patients	111		Treatment with SP
Sex			
Male (%)			59 (53%)
Females (%)			52 (47%)
Male:female ratio			1.13
Age (Years)			
Mean±SD			4.19±2.67
Range			1-12
n (<5 years)			79
n (>5 years)			32
Parasites count (/μl)			
Geometric mean			25,963
Range			1090-449,143
Proportion of patients with > 100,000			21% (23/111)
Proportion of patients with < 100,000			79% (88/111)

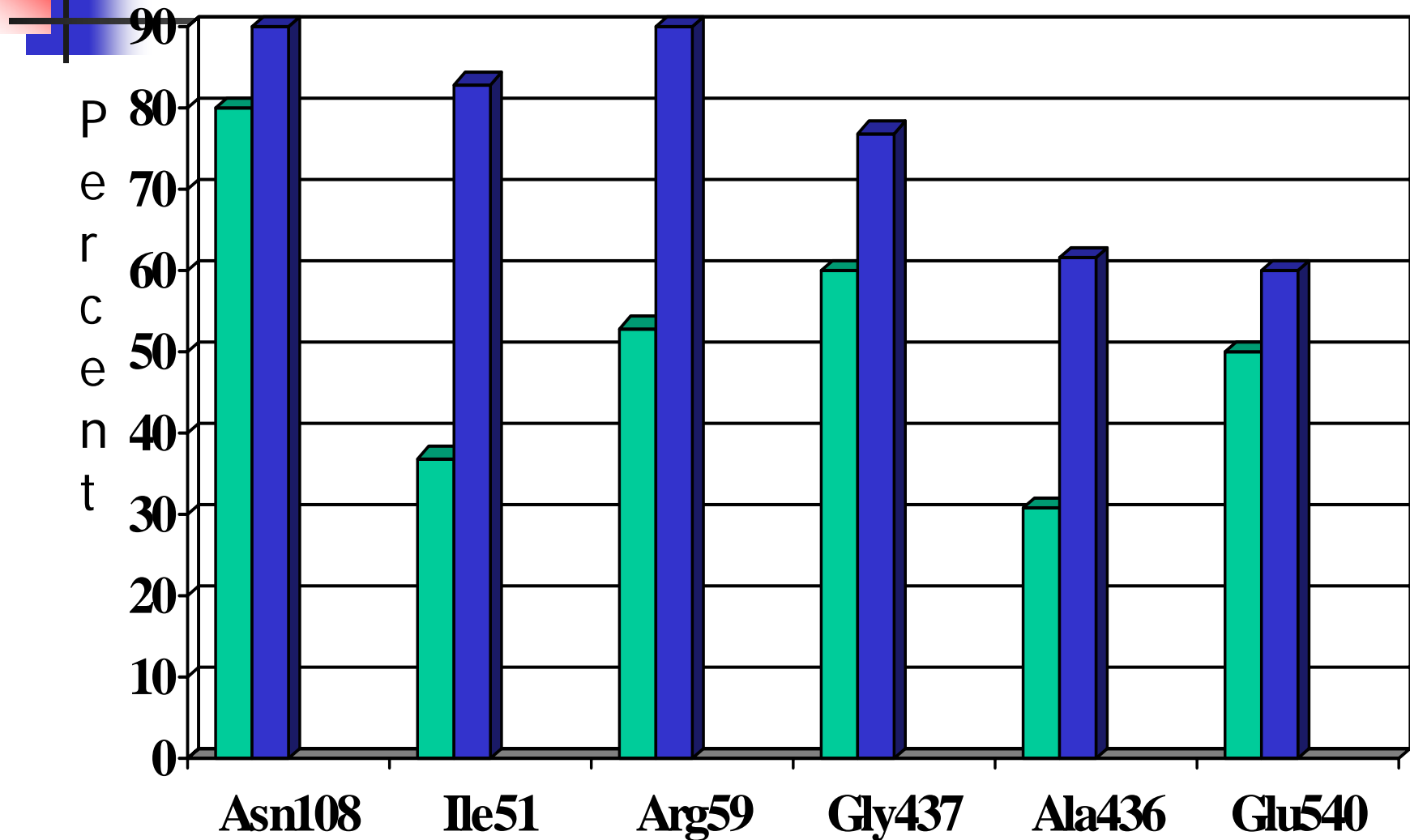
Treatment outcomes of malaria infected children with SP.



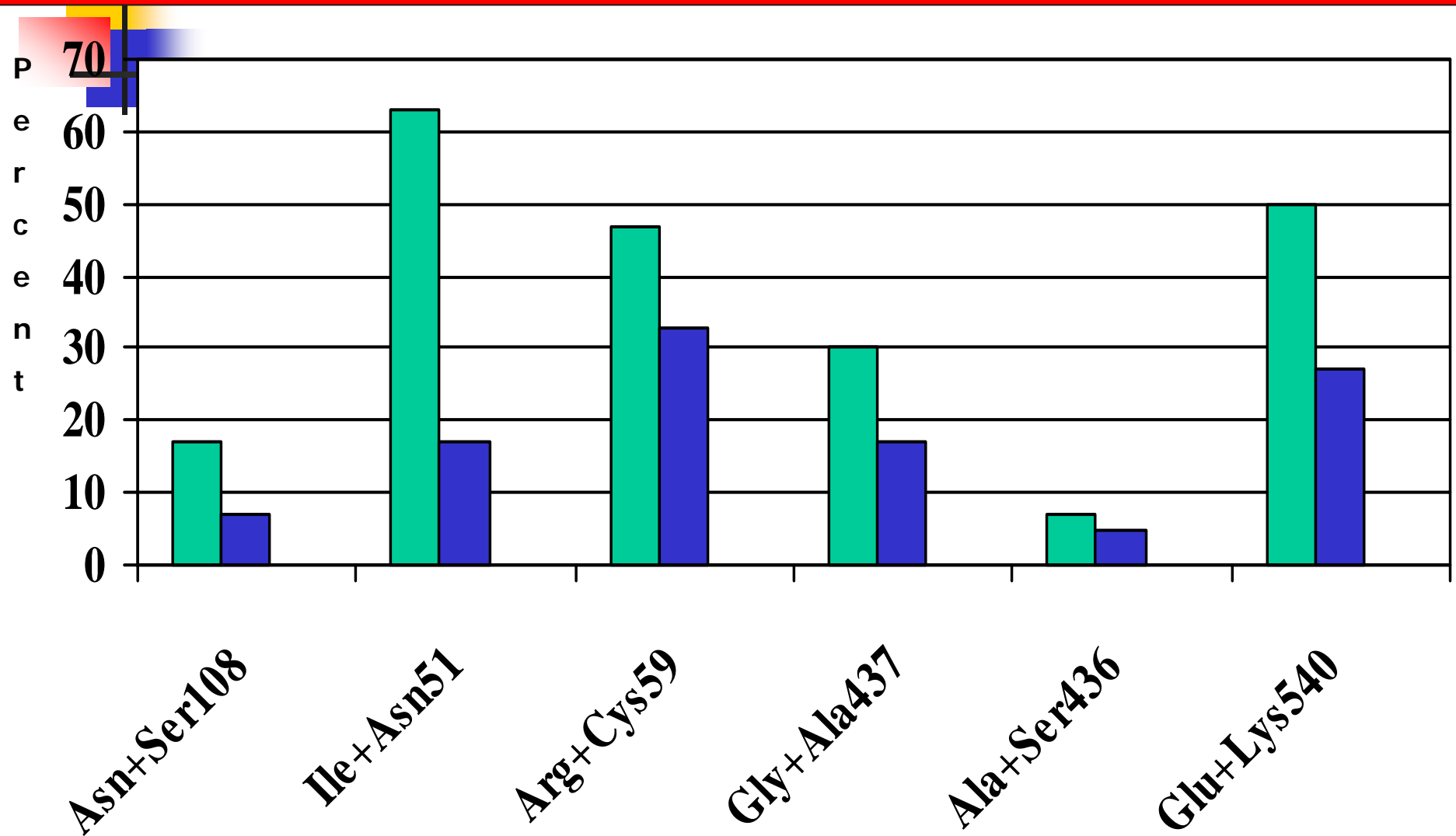
Prevalence of *dhfr* and *dhps* mutations at enrollment in isolates of *P. falciparum* infections from Ibadan, Nigeria. The mutations detected by PCR and RFLP assays.



Prevalence of polymorphisms in *DHFR* and *DHPS* genes in **Pre-** and **Post-**treatment isolates obtained from patients who failed sulfadoxine-pyrimethamine.



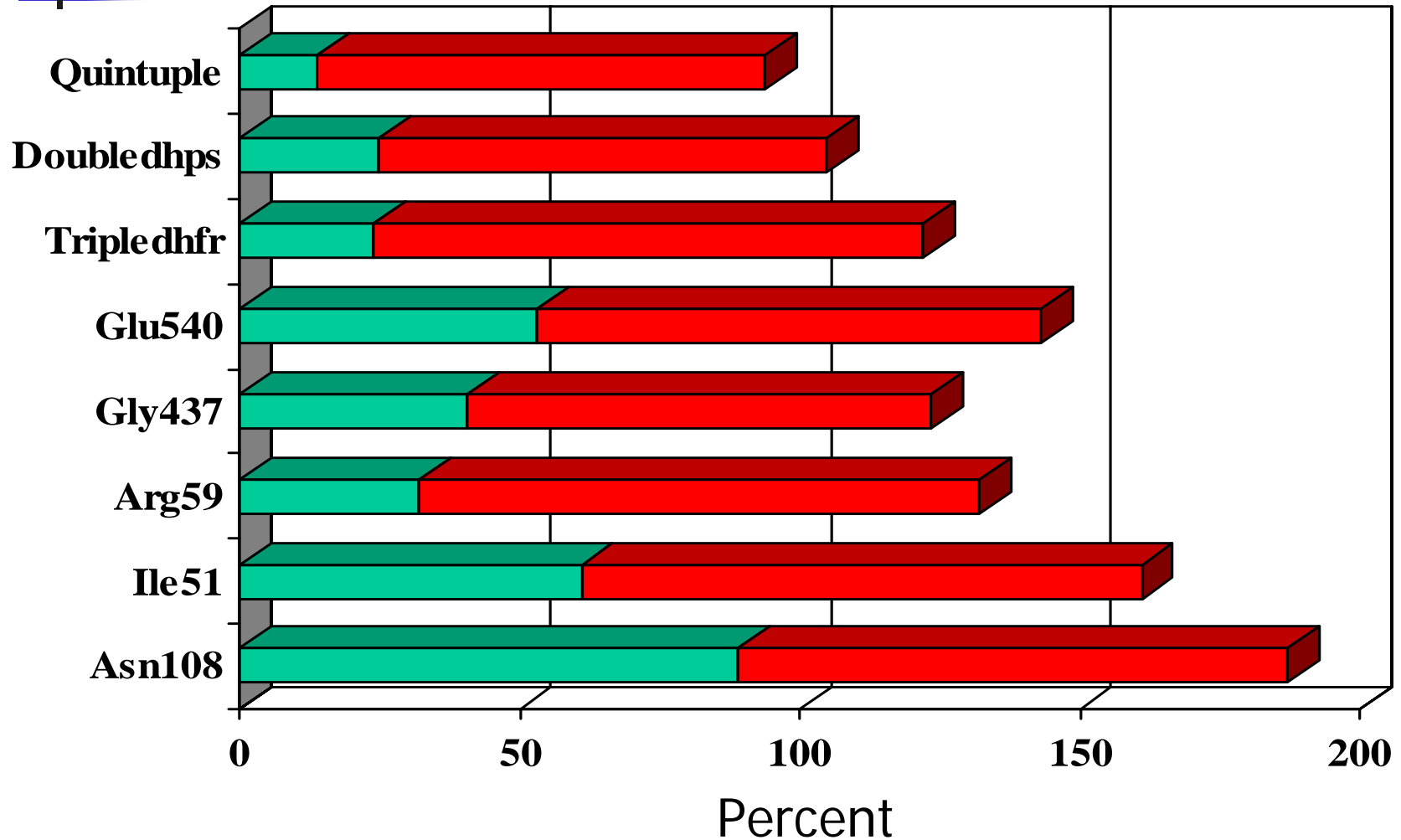
Prevalence of mixed polymorphisms in *DHFR* and *DHPS* genes in **Pre-** and **Post-**treatment isolates obtained from patients who failed sulfadoxine-pyrimethamine.



Prevalence of polymorphisms in *dhfr/dhps* genes in pre-treatment samples obtained from patients on SP treatment.

<i>dhfr/dhps</i> mutations	Treatment outcome, no. of patients (%)		OR (95% CI)	p-value
	Cured	Failed		
<i>Dhfr</i> Asn-108	70 (89%)	29 (98%)	3.72 (0.452-30.78)	0.279
<i>Dhfr</i> Ile-51	48 (61%)	30 (100%)	1.6 (1.37-1.96)	0.000
<i>Dhfr</i> Arg-59	25 (32%)	30 (100%)	3.1 (2.28-4.37)	0.000
<i>dhps</i> Gly-437	32 (40.5%)	25 (83%)	7.4 (2.54-21.19)	0.000
<i>dhps</i> Glu-540	42 (53%)	27 (90%)	7.92 (2.22-28.29)	0.000
Triple <i>dhfr</i> mutant Asn-108/Ile-51/Arg-59	19 (24%)	29 (98%)	9.57 (11.68-717.92)	0.000
Double <i>dhps</i> mutant Gly-437/Glu-540	20 (25%)	24 (80%)	8.11 (3.12-21.08)	0.000
Quintuple <i>dhfr/dhps</i> mutant*	11 (14%)	24 (80%)	24.72 (8.24-74.15)	0.000


Association between *dhfr* and *dhps* mutations in *P.falciparum* isolates collected at enrollment and SP treatment
(Cured & Failed)



Selection of dhfr mutations by SP

<i>dhfr/dhps</i> alleles at different codons	Prevalence of point mutations in patients samples (%)		p-value
	Enrollment	Recrudescence	
<i>dhfr</i> Codon 108			0.27
Wild-type Ser (AGC)	3%	3%	
mutant Asn (AAC)	80%	90%	
Mixed Ser+ Asn	17%	7%	
<i>dhfr</i> Codon 51			0.0100*
Wild-type Asn (AAT/AAC)	-	-	
mutant Ile (ATT)	37%	83%	
Mixed Asn+Ile	63%	17%	
<i>dhfr</i> Codon 59			0.003*
Wild-type Cys (TGT)	-	-	
Mutant Arg (CGT)	53%	90%	
mixed Cys+Arg	47%	33%	
<i>dhps</i> Codon 437			0.369
Wild-type Ala (GCT)	10%	6%	
Mutant Gly (GGT)	60%	77%	
mixed Ala+Gly	30%	17%	
<i>dhps</i> Codon 540			0.227
Wild-type Lys (AAA)	10%	13%	
Mutant Glu (GAA)	50%	60%	
mixed Lys+Glu	40%	27%	

Impact of age and *dhfr* / *dhps* mutations on patients treatment outcome

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- Age-stratified analysis showed that the presence of *dhfr* and *dhps* quintuple mutations significantly predicted the risk of SP treatment failure in both age groups (P=0.000 & P=0.002).
 - The association between single *dhfr* or *dhps* mutations and treatment failures were significant only in children <5 years (P<0.05), except for the *dhfr* Asn-108 in both age groups.
 - There were no significant relationship between the *dhfr* triple mutants or *dhps* double mutant and treatment failures in children >5 years old (P=0.19).



Conclusions (1)

- The high prevalence of *dhfr* or *dhps* mutations in *P. falciparum* isolates from malaria infected children as well as the 27% *in vivo* resistance to SP in Nigeria is of great concern.
- The *dhfr* (Asn-108, Ile-51, Arg-59) / *dhps* (Glu-540, Gly-437) quintuple mutant is the most reliable subset of predictor of SP treatment failure in *P. falciparum* isolates from Nigeria.
- Age-acquired immunity is a major factor in clearing SP resistant parasites during treatment with SP in moderate or high malaria areas.

Conclusion (2)



- Considering the prevalence of *dhfr/dhps* quintuple mutant (29%) and the 28-day treatment failure rate of 27%, the adjusted Genotype Failure Index (GRI) value in Ibadan, Nigeria was equal to 1.07.
- Further work is needed to validate the age- adjusted GRI predictive model of SP treatment failure as proposed in this study in different epidemiological settings.



Acknowledgements

Team:

- Prof. Sowunmi A.
- Dr. Grace Gbotosho
- Dr. Christian Happi
- Mrs. O.A. Folarin
- Mr. Ernest Tambo
- Mrs Amoo (Nurse)
- Prof. A.M.J. Oduola (WHO-TDR)
- MR4.
- WHO/MIM/TDR project ID A20239
- Prof. Dyann Wirth Harvard Malaria Initiative (HSPH, Boston, MA)
- FIRCA Grant No. NIH RO3TW006298-01A1