

Association Between Sickle Cell Trait and Low Density Parasitaemia Among Asymptomatic and Parasitaemic individuals Living in *P. falciparum* Malaria Holoendemic Region of Western Kenya.

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Abstract

The frequency of the mutant gene for sickle cell is widely distributed in the sub-Saharan Africa, the Middle East and the Indian subcontinent. There is epidemiologic evidence that sickle cell trait confers a survival advantage against malaria and that the selection pressure due to malaria has resulted in high frequencies of the mutant gene in areas of high malaria transmission. Kisumu West District has been used for a number of vaccine trials and other epidemiological studies. We carried out a study to look at the relationship between sickle cell trait, age, haemoglobin level and malaria parasite density.

Methods

We carried out a cross-sectional study between the months of October and December, 2004 in Kombewa Division of Kisumu West District, a *P. falciparum* malaria holoendemic area with entomological inoculation rates estimated at 31.1 infective bites per person per year. We screened and quantified malaria parasitaemia in participants (age 0 to 45 years n = 342). Haemoglobin electrophoresis was performed on blood from all the participants.

Results

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In total, 402 participants were screened of which 342 were enrolled. Of these, 280 (81.4%) had haemoglobin AA, 60 (17.4%) had haemoglobin AS and 2 (0.6%) had haemoglobin SS. Those with HbAA and HbAS were included for the analysis bringing the total number to 340 participants. For asymptomatic individuals in the community who displayed no signs of an acute or chronic illness and who were *P. falciparum* malaria parasite positive; the mean parasite density/ μL for HbAS of 4064.0 (95% CI 1858.0 – 6270.0) was significantly lower than that of HbAA 11,067.9 (95% CI 7616.0 – 14520.0) ($P = 0.001$).

Conclusion

The sickle cell carrier status is high (17.4%) in this population and is protective against high density parasitaemia. It is suggested that any malaria intervention strategies should factor in the possibility of sickle cell trait as a confounder to the protective effect of the intervention.

Introduction

P. falciparum malaria has had a huge impact on the human genome as evidenced by the relative resistance to malaria through numerous hereditary red cell disorders including membrane protein disorders, enzyme deficiencies and haemoglobinopathies (Durant PM & Coetzer TL, 2008). Sickle cell disease refers to a collection of autosomal recessive genetic disorders characterized by the presence of HbS variant of the globin chain (Ashley-Koch *et al.* 2000) while sickle cell trait (HbAS) is the heterozygous form of sickle cell disease where the individual has normal haemoglobin HbA (normal) and HbS (sickle cell) gene. HbS carriers are protected from malaria infection and this is thought to have led to high frequency of HbS in individuals of African and Mediterranean ancestry (Ashley-Koch *et al.* 2000). Haldane (1949) observed that several red blood cell disorders such as sickle cell anaemia are common in areas where malaria was endemic and hence hypothesized that malaria was the selection pressure for the occurrence of some of these genetic polymorphisms. A recent investigation by Crompton *et al.* (2008) reported that HbAS was associated with a delay in time to first malaria episode between the ages of 2-10 years. It has been suggested that protection from malaria by HbAS involves enhancement of both innate and acquired immunity to the parasite (Williams TN *et al.* 2005a). The protective effect of HbAS was found to be remarkably specific for *P. falciparum* malaria (Williams TN *et al.* 2005b) which is the major cause of devastating illness especially in the paediatric age group (Marsh K *et al.* 1995). Williams *et al.* (2005b) however found out that HbAS had no effect on the prevalence of symptomless parasitaemia but was protective against mild clinical malaria, admission to hospital for malaria and approximately 90% protective against severe or complicated malaria. Although malaria is

often fatal in individuals with sickle cell anaemia (HbSS), the protection from infection appears to operate in HbS dose dependent manner and individuals with HbSS have an even lower risk of infection than those with HbAS (Aluoch JR 1997). The area where this study was done has been used for several epidemiological and vaccine trials and is currently one of the sites for an ongoing malaria vaccine trial and therefore it was important to know the level of sickle cell carrier status and the protective effect of sickle cell trait against high density malaria parasitaemia. This was part of a cross-sectional study entitled “Erythrocyte Immune Complex Binding Capacity and Complement Sensitivity in Populations with Different Malaria Risks”. As part of this study, we compared participants with HbAS and HbAA to see whether there were any differences in the haemoglobin levels, the presence of malaria parasitaemia and parasite densities between the two groups during the period participants were well, a febrile and had no signs of any acute or chronic illness.

Materials and Methods

Study site

The study site was Kombewa Division, in Kisumu West District, Nyanza Province of western Kenya. It is situated about 35 kilometres west of Kisumu town and has previously been used as a site for many epidemiological studies in both adults and children (Kifude CM *et al.* 2007; Ndenga B *et al.* 2006)

Kombewa borders Lake Victoria and has a population of about 65 000 people. Malaria transmission in this area occurs all year round with peak seasons following the long rains (March to May) and the short rains (October to December). The annual inoculation rates are estimated to be 31.1 infective bites per person per year (Ndenga B *et al.* 2006).

Study Design and Patient Population

Healthy male and females aged between 0 to 45 years and residents of Kombewa Division of Kisumu West District were recruited. Since many acute or chronic conditions including malaria (Roestenberg M *et al.* 2007; Stoute JA *et al.* 2003; Stoute JA 2005; Waitumbi JN. *et al.* 2000), HIV infection (Aries SP *et al.* 1997) and others (Spendlove I *et al.* 2006), are known to interfere with complement regulatory proteins, the main subject of focus for the current study, the exclusion criteria included evidence of malnutrition, immuno compromised states, severe anaemia (haemoglobin \leq 5.0 g/dl); bacterial infection such as pneumonia; malignancy; and blood transfusion within 3 months preceding the study. In cases of an acute illness, the

potential participants were assessed, treated and asked to come again for re-evaluation. At re-evaluation, the potential participants were enrolled when they were deemed well.

Recruitment

All subjects underwent a standardized clinical evaluation and physical examination by an experienced physician or physician assistant at enrollment. Blood was collected after written informed consent. Minor illnesses such as upper respiratory tract infections were treated and if malaria was present, it was also treated. All subjects with uncomplicated malaria were given treatment with Artemether/lumefantrine (Coartem®). Individuals who were still parasitaemic following treatment with Coartem were treated with quinine. Participants were excluded when they had evidence of concomitant infection, severe malaria and other conditions which in the opinion of the clinician could affect the parameters being measured as described above.

Blood Sample Collection and Processing

Whole blood for haemogram and haemolysate preparation was collected by trained clinical personnel using sterile and disposable needles, syringes or lancets into a 2 mL EDTA vacutainer tube (Becton Dickinson, San Diego, CA) and kept at 4°C until processed. Complete blood count was measured using a standard haematology analyzer (Coulter, Hialeah, FL).

Assays and Procedures

Blood Smears

At enrollment, approximately 10 µl of blood was used for preparation of Giemsa-stained thick and thin smears from finger prick or EDTA blood collected by venipuncture. After staining, blood smears were read by experienced microscopists to confirm the diagnosis of *P. falciparum* malaria. A minimum of 50 high power fields (HPF) were scanned for a positive smear and 200 HPF for a negative smear. The number of asexual stage parasites was presented per 200 white blood cells (WBCs) counted. The parasite density per µL was calculated by multiplying the total WBCs by the number of parasites per 200 WBCs.

Haemoglobin Electrophoresis

Haemoglobin electrophoresis on cellulose acetate plates was carried out under alkaline conditions using reagents and kits from Helena Laboratories (Beaumont, TX). Briefly, 100 µL of EDTA whole blood was placed in a micro-centrifuge tube and the packed red cell pellet were washed three times with phosphate buffered saline (PBS), pH 7.4. Packed cells (10µL)

was mixed with 60 μ L of hemolysate reagent, mixed well by vortexing and allowed to stand for 5 minutes. Test samples and controls (5 μ L each) were placed on each well of the electrophoresis chamber. After applying voltage for 25 minutes, the plate was soaked in Ponceau S stain for 5 minutes and destained in successive washes of 5% acetic acid for 2 minutes.

Statistical analysis

Statistical analyses were performed using SPSS for windows version 11.5 and 15.0 software (SPSS Inc, Chicago, IL, USA). The parasite density data were presented graphically for each age group as box plots, where the box represented boundaries between the 25th and 75th percentile, the line through the box represented median and whiskers the 10th and 90th percentile limits. Analysis of variance (ANOVA) was used to detect differences across age groups adjusting for factors and covariates. The independent samples t-test was used for comparisons of normal continuous data between two groups. Chi-square (χ^2) and Mann-Whitney U tests were used to examine differences between proportions and for pairwise comparisons of medians, respectively. Bivariate logistic regression analysis was used to determine the Odds Ratio (OR) and the mean and standard error of mean (SEM) for haemoglobin level and the presence of *P. falciparum* parasitaemia. The chi square test was used to compare proportions across groups. All tests were two-sided with $\alpha \leq 0.05$.

Ethical consideration

The recruitment of human subjects and study procedures were in accordance with all applicable regulations. Informed consent was obtained from all participants or parents/guardians of children. This study was reviewed and approved by the Kenya National Ethical Review Committee and by the Human Subjects Research Review Board of the Office of the Surgeon General, U.S. Army.

Results

Recruitment of Volunteers

Four hundred and two (402) volunteers were screened of whom 190 were enrolled on the first visit. 196 volunteers had malaria and other illnesses. They were treated and asked to return after two weeks while 16 were excluded for various reasons based on the exclusion criteria. During the second visit, 152 volunteers were enrolled, 10 were given treatment a second time and 34 were lost to follow up. Three volunteers were enrolled on the third visit and 7 were

excluded after failing to meet the recruitment target. Haemoglobin electrophoresis was carried out on the enrolled target population.

Demographic and Laboratory Characteristics

Three hundred and forty two (342) participants were enrolled for this study of which, 280 (81.4%) had haemoglobin AA, 60 (17.4%) had haemoglobin AS and 2 (0.6%) had haemoglobin SS. For analysis individuals with HbAA and HbAS were included. Results presented here show that gender ($P = 0.828$), median age in months ($P = 0.726$) and presence or absence of malaria parasitaemia ($P = 0.638$) were comparable between the two groups. Mean parasite count/ μL for the HbAA was 4822.4 (95% CI 3196.30 – 6448.60), more than twice as high as those with HbAS 1693.3 (95% CI 669.70 – 2717.00). However bivariate analysis using Mann-Whitney U test revealed no statistical significance ($P = 0.406$), see Table 1 below.

Table 1: Demographic and Laboratory Characteristics of the Study Participants

Characteristic	HbAA (n =280)	HbAS (n = 60)	P
Gender			
Male n (%)	145 (51.8)	32 (53.3)	0.828 ^a
Female n (%)	135 (48.2)	28 (46.7)	
Age in months (Median; 25th -75th percentiles)	36.50 (14.00 – 130.25)	30.0 (12.25 – 128.25)	0.726 ^b
Haemoglobin levels (Median; 25th -75th percentiles)	11.00 (9.80 – 12.58)	11.15 (10.23 – 12.10)	0.825 ^b
Mean parasite density (95% C I)	4822.4 (3196.30 – 6448.60)	1693.3 (669.70 – 2717.00)	0.406 ^b
Numeric <i>P. falciparum</i> read			
Negative n (%)	154 (55.0)	35 (58.3)	0.638 ^a
Positive n (%)	126 (45.0)	25 (41.7)	

Data are presented as median (25th – 75th percentiles) unless otherwise stated. ^astatistical significance determined by the Chi-square (χ^2) analysis, ^bstatistical significance determined by Mann-Whitney U test

Laboratory Characteristics of individuals with Parasitaemia

For participants with HbAS and parasitaemia, there was no significant difference in gender ($P = 0.326$), median age in months ($P = 0.707$) and median haemoglobin levels ($P = 0.602$). The mean parasite density/ μL for HbAS was 4064.0 (95% CI 1858.0 – 6270.0) and for the HbAA

the parasite density/ μL was 11,067.9 (95% CI 7616.0 – 14520.0). This difference was statistically significant $P = 0.001$ (independent sample T-test) see Table 2.

Table 2: Demographic and Laboratory Characteristics of individuals with parasitaemia within the Study Groups

Characteristic	HbAA (n = 122)	HbAS (n = 25)	P
Gender			
Male n (%)	61 (50.0)	15 (60.0)	0.326 ^a
Female n (%)	61 (50.0)	10 (40.0)	
Age in months (Median; 25th -75th percentiles)	37.50 (13.00 – 104.50)	30.00 (10.00 – 95.50)	0.707 ^b
Haemoglobin levels (Median; 25th -75th percentiles)	11.00 (9.86 – 12.50)	11.30 (10.60 – 11.85)	0.602 ^b
Mean parasite density (95% C I)	11,067.87 (7615.95 – 14519.79)	4,064.00 (1857.93 – 6270.07)	0.001 ^c

Parasitemic children (n=147) were stratified according to their haemoglobin type into (a) HbAA (n=122) and (b) HbAS (n=25). Data is presented as either mean or median with appropriate intervals or percentiles respectively. a - χ^2 analysis, b – Mann-Whitney U test, c – Independent sample T-test

Haematological and Parasitological Characteristics of the Study Cohorts

Study participants were divided into 8 age group cohorts. The mean haemoglobin level, haemoglobin type, and percentage of those who had malaria parasitaemia in each of the age cohorts are summarized in Table 3 and Figures 1, 2 respectively.

Table 3: Haematological and Parasitological Characteristics of the Study Groups

Age Cohort (Months)	Nr.	Haemoglobin Level	Haemoglobin AS (%)	<i>P. falciparum</i> positive (%)
0 – 6*	31	11.6 (10.6 – 11.8)	9.7	19.4
7 – 12*	60	10.1 (9.8 – 10.5)	18.3	33.3
13 – 24	60	9.6 (9.2 – 10.0)	18.3	60.0
25 – 48	60	10.6 (10.2 – 11.0)	16.7	55.0
49 – 96	31	12.3 (12.0 – 12.7)	16.1	71.0
97 – 192	30	12.7 (12.2 – 13.2)	56.7	70.0
193 – 384	42	13.4 (12.7 – 14.0)	9.5	28.6
385 – 540	30	12.9 (12.1 – 13.6)	30.0	13.3

*Two participants in the 0 – 6 and 7 – 12 months age categories had haemoglobin SS.

Out of the 344 participants, 14 had *P. ovale* and 24 had *P. malariae* infections

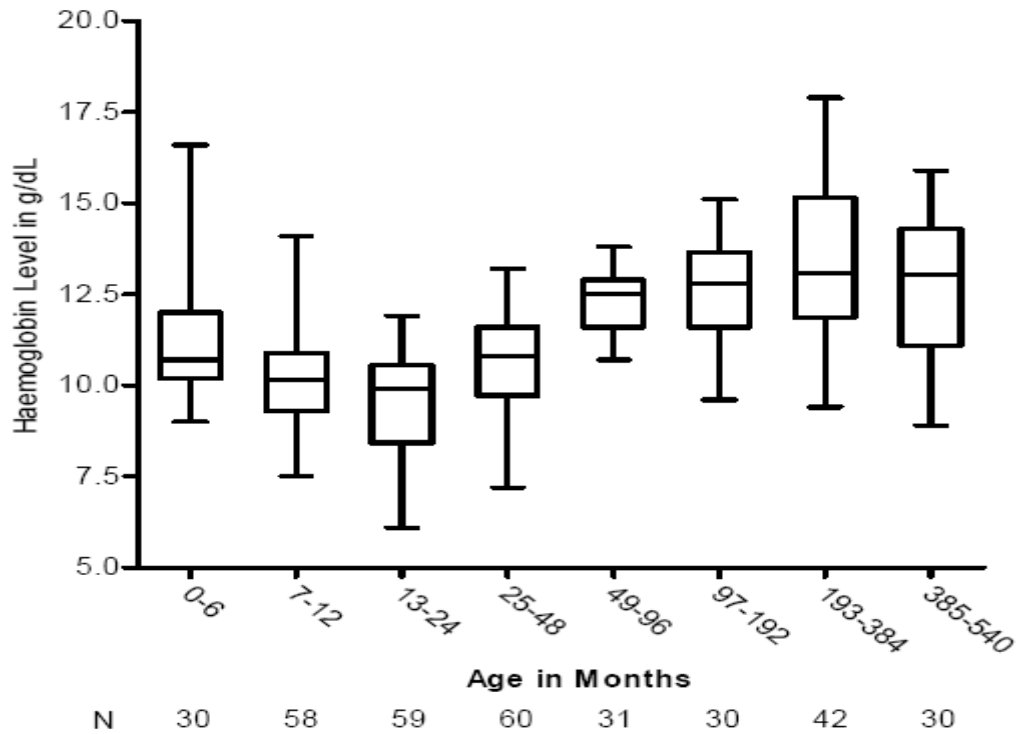


Figure 1: Mean haemoglobin levels for the age cohorts

Of the 196 volunteers who had malaria at first visit, majority were children in the >24-48 (36/60), >48-96 (33/60) and >96-192 (22/31) months age category with the highest percentage in the >48-96 month age category. Despite many participants being parasitaemic in the >48-96 month age category, the mean parasite density/ μL was highest in the >12-24 months category (Figure 2).

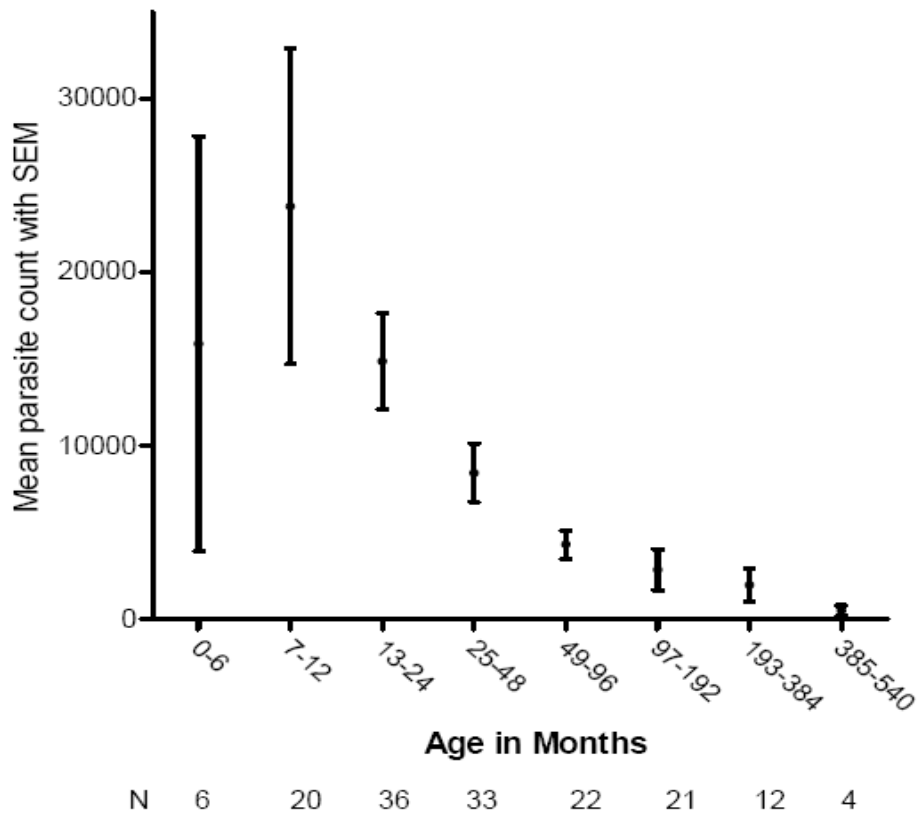


Figure 2: Mean *P. falciparum* parasite count/ μ L in the age cohorts

Discussion

The mortality caused by malaria in high malaria endemic parts of the world has led to the evolution of sickle cell trait which has been shown to protect against severe manifestations of malaria [6]. Haldane proposed that the sickle cell gene provides an advantage in malaria endemic regions [3]. Allison AC 1954, presented evidence for the balanced polymorphism of sickle cell gene and the sickle cell trait. Sickle cell carrier status provides resistance to *P. falciparum* malaria (Bayoumi *et al.* 1986). Sickle cell trait has been shown to lead to delay in the first malaria episode [4], which would suggest that haemoglobin typing ought to be done as part of the clinical malaria vaccine and drug trials. This study showed that the mean parasite density/ μ L for HbAS was 4064.0 (95% CI 1858.0 – 6270.0) and for the HbAA, the mean parasite density/ μ L was 11,067.9 (95% CI 7616.0 – 14520.0). This difference was statistically significant ($P = 0.001$, Independent sample T test). This study is thus, in agreement with an earlier study which was done in a malaria holoendemic area of Benin (Chippaux JB, 1992). In the Benin study, the mean *P. falciparum* parasitaemia were

significantly lower in HbAS compared to HbAA children and sickle cell trait seemed to decrease the level of parasitaemia ((Chippaux JB, 1992). Furthermore, other studies have showed that individuals with HbAS have lower parasite densities compared to HbAA individuals irrespective of their malaria status(Williams TN *et al* 2005; Aidoo 2002) and indeed, the time to reappearance of *P. falciparum* is shorter in HbAS individuals after drug treatment (Sohna t al 2000). The lowest level of haemoglobin for various age cohorts was in the >12-24 months age category which also corresponded to high increase in the percentage parasitaemia. In keeping with previous reports (Crawley J 2004; Eliades MJ 2006), a significant correlation was observed between age and haemoglobin level $r(340) = 0.550$ $P = 0.0001$. This low peak in haemoglobin level has recently been associated with increasing malaria prevalence; low complement regulatory proteins, increased C3b deposition and therefore development of malaria irrespective of the carrier status for sickle cell (Odhiambo CO *et al.* 2008)

Conclusion

Sickle cell carrier status is prevalent in this region (17.4%) and this study has shown that in the presence of carrier status for sickle cell (HbAS), these children are protected from getting high density malaria parasitaemia. Given the various epidemiological, drugs and malaria vaccine trial and the fact that some studies have also shown similar results (Bayoumi *et al.* 1986; Sokhna CS *et al.* 2000), it is important to screen all potential volunteers in these studies for sickle cell trait and factor this in the analysis so as to know the protective role from sickle cell trait when compared to that offered by these other interventions. The lowest level of haemoglobin in the community was at the age of 13 -24 and this corresponded to the period where these children have the highest mean parasite counts. This may explain the high prevalence of anaemia in this age group.

Recommendations

This study has shown that HbAS is prevalent in Kisumu west district and that in the presence of HbAS; children are protected from getting high density malaria parasitaemia. Given the various malaria drug trials and the ongoing and future malaria vaccine trial and the fact that some studies have also shown similar results (Sokhna CS *et al.* 2000), it is important to screen all potential study volunteers for sickle cell trait and factor this in the analysis so as to know the protection from sickle cell trait against protection from the various malaria

interventions because HbAS can be a confounder in determining efficacy of both the various drug trials and malaria vaccine.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

WO helped in study design, conducted all experiments, helped in data analysis and drafted the manuscript, BBAE helped in study design, data analysis, data interpretation and helped in drafting of the manuscript, JRA helped in study design, data analysis, data interpretation and helped in drafting of the manuscript, SMOG helped in study design, data analysis, data interpretation and helped in drafting of the manuscript.

Disclaimer

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