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Short Report: G6PD A- Deficiency and Severe Malaria in The Gambia: Heterozygote Advantage and Possible Homozygote Disadvantage

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Abstract. Glucose-6-phosphate dehydrogenase (G6PD) deficiency is frequent in Africa, because it confers resistance to *Plasmodium falciparum* malaria; however, the nature of the protection and the genotypes associated with it have been controversial. In 1972, Bienzle and others described protection from malaria in West African females heterozygous for G6PD A-. They determined that G6PD A- heterozygotes had lower parasite counts than A- homozygotes, hemizygous males, and normal individuals. However, other studies have reached different conclusions about the protective genotypes. DNA samples from 135 children with severe malaria and 146 children with mild malaria from The Gambia were genotyped for the G6PD A- mutation that is most frequent among Gambians (*G6PD* 968 T->C); there was a marked deficiency of heterozygotes and an excess of homozygotes with severe malaria, producing a strong deviation from Hardy–Weinberg equilibrium. Our results support the protective effect in G6PD A- heterozygous females and suggest that homozygotes might be more susceptible to severe malaria attacks.

Glucose-6-phosphate dehydrogenase (G6PD) deficiency is widespread across Africa, because it confers resistance to *Plasmodium falciparum* (and possibly, *P. vivax*) malaria, but despite this evidence and the previous evidence that *G6PD* is under strong natural selection, the nature of the protection and genotypes associated with it has been controversial.[1–3] In 1972, Bienzle and others[4] described protection from malaria in West African females heterozygous for G6PD A-; their study was based on 255 boys and 203 girls with *P. falciparum* malaria recruited from a holoendemic area in southwestern Nigeria. Bienzle and others[4] determined that G6PD A- heterozygotes had significantly lower parasite counts than A- homozygous females, hemizygous males, and normal individuals. Since the report by Bienzle and others,[4] other studies have had conflicting results, including a recent conclusion by Guindo and others[5] based on a Malian sample of children with severe malaria that hemizygous males and possibly, homozygous but not heterozygous females are protected from disease. The argued protection in hemizygous males was supported by pooled odds ratios from meta-analysis of their data[5] "and data from a previous study"[6] that "confirmed highly significant protection against severe malaria in hemizygous males but not in heterozygous females." However, because the "previous study" that Guindo and others[5] referred to was based on largely problematic genotyping data,[2,6,7] their meta-analysis is also questionable.

The most common enzyme-deficient African variant, A-, has 12% of the normal enzymatic activity, and its frequency is as high as 0.24 in Nigerian Yoruba.[8–10] G6PD A- is molecularly characterized by two distinct variants within the gene: one variant is always 376 A->G (underlying G6PD A, which has 85% enzyme activity and is not considered to be a deficient variant); a second deficiency-causing mutation is 202 G->A (376G/202A; most common), 680 G->T (376G/680T), or 968 T->C (376G/968C; Betica Selma).[11] Virtually all of the second A- mutations have been found in the presence of 376 A->G, with only one exception reported so far.[12]

Although the 202 G->A mutation is largely responsible for G6PD A- deficiency in much of sub-Saharan Africa, in The Gambia (and likely, the whole of Senegal[13]), the major mutation causing A- is 968 T->C, with a frequency of 7%, whereas 202 G->A is only 2–3%; therefore, approximately 10% (7% + 3%) of Gambian males are G6PD A-.[14] An additional mutation, 542 A->T (376G/542T), that causes a severe deficiency with 2% residual activity (G6PD Santamaria) occurs in about 2% of Gambian males.[14] Thus, the overall frequency of G6PD deficiency in The Gambia is approximately 12%.

To address the role of enzymatic deficiency, we analyzed *G6PD* 202 G->A and 968 T->C genotypes in Gambian children with malaria (age range = 6 months to 16 years), because this combination accounts for most of the deficiency in The Gambia.[15] Subjects for our study were enrolled into a health center-based case-control study that has been described elsewhere.[15] Subjects were clinically assessed by a research physician and stringently classified as children with uncomplicated (mild) malaria (N = 146; 63 females and 83 males) and severe malaria (N = 135; 60 females and 75 males) resident in a restricted periurban area. Mild malaria was defined as an episode of fever (temperature > 37.5°C) within the last 48 hours and more than 5,000 parasites/μL detected by slide microscopy. Subjects were, to the best of our knowledge, unrelated. Relatedness is unlikely given that our samples were drawn from different villages in the greater Banjul area (within 40 km of the city) and several ethnic groups. The study was reviewed and approved by the Gambian Government/Medical Research Council (MRC) Joint Ethics Committee. All patients were enrolled after written informed consent was obtained from the parents or guardians.

In addition to the above criteria, severe malaria was defined by the presence of one or more of the following criteria: severe anemia (SA; defined as hemoglobin [Hb] < 6 g/dL); severe respiratory distress (SRD; defined as serum lactate > 7 mmol/L); cerebral malaria (CM; defined as a

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TABLE 1

Clinical presentation of severe malaria cases

	CM-SA-SRD	CM-SRD	SRD-SA	CM-SA	CM	SA	SRD	SP	Total
Total	1	11	1	0	18	11	16	77	135
No. of males (%)	1 (1.33)	5 (6.67)	1 (1.33)	0 (0.00)	11 (14.67)	5 (6.67)	10 (13.33)	42 (55.00)	75
No. of females (%)	0 (0.00)	6 (10.00)	0 (0.00)	0 (0.00)	7 (11.67)	6 (10.00)	6 (10.00)	35 (58.33)	60

CM = cerebral malaria; SA = severe anemia; SRD = severe respiratory distress; SP = severe prostration. Percentages are the proportions of the samples among the different phenotypes of severe malaria.

TABLE 2

List of primers

Variant	Primer forward	Primer reverse	Extension primer
202 G->A	ACGTTGGATGTCACTCTG TTTGCGGATGTC	ACGTTGGATGCCTTCTGCCC GAAAACACC	CGGGAACGGGCATAGCCCA
376 A->G	ACGTTGGATGTAGAAGA GGCGGTTGGCCTG	ACGTTGGATGATGATGCAG CCTCCTACCAG	CCCCAGGTGGAGGGCAT
968 T->C	ACGTTGGATGCATTCTCT CCCTTGGCTTTC	ACGTTGGATGACCACATTG TTGGCCTGCAC	CTCTCAGGTCAAGGTGT

TABLE 3

968 T->C genotypes of females

	Mild ($N = 63$)		Severe ($N = 60$)	
Females	Observed	Expected	Observed	Expected
TT	55 (0.87)	55.30	53 (0.88)	49.50
CT	8 (0.13)	7.50	3 (0.05)	10.00
CC	0 (0.00)	0.30	4 (0.07)	0.50
Hardy–Weinberg *P* value	0.59		< 0.0001*	

* $P = 0.01$ after Monte Carlo permutation test (10,000 runs).

TABLE 4

968 T->C genotypes of males

Males	Mild observed ($N = 83$)	Severe observed ($N = 75$)
T	82 (0.99)	73 (0.97)
C	1 (0.01)	2 (0.03)

TABLE 5

Freeman–Halton extension of Fisher's exact (two-tailed) probability

Malaria type	C or CC	C/T	T or TT	Total
Severe	6	3	126	135
Mild	1	8	137	146
Total	7	11	263	281

Exact probability: 0.048.

TABLE 6

Logistic regression results: odds of being mild—model 968 T->C (both sexes)

Model	OR	95% Confidence interval	*P* value
CC or C vs. TT or T	6.52	0.78–54.89	0.085
CT vs. CC or C	0.06	0.005–0.76	0.030
CT vs. TT or T	0.41	0.11–1.57	0.192

TABLE 7

Logistic regression results: odds of being mild severe—model 968 T->C and sex (non-additive)

Model	OR	95% Confidence interval	*P* value
968T->C and sex (non-additive)			
Sex	1.12	0.69–1.83	0.683
968 T->C			
CC or C vs. TT or T	6.41	0.76–54.04	0.088
CT vs. CC or C	0.06	0.01–0.73	0.027
CT vs. TT or T	0.38	0.10–1.51	0.171

OR is determined in comparison to the referent genotype, which is given on the left of the column.

TABLE 8

Logistic regression results: odds of being mild—model 968 T->C, sex, and sickle status (non-additive)

Model	OR	95% Confidence interval	*P* value
968T->C, sex, and sickle status (non-additive)			
Sex	0.97	0.57–1.65	0.907
Sickle status	1.74	0.38–7.98	0.477
968 T->C			
CC or C vs. TT or T	4.89	0.56–42.58	0.157
CT vs. CC or C	0.08	0.01–0.96	0.047
CT vs. TT or T	0.38	0.09–1.49	0.163

Blantyre coma score ≤ 2 in the absence of hypoglycemia or hypovolemia, with the coma lasting at least 2 hours); severe prostration (SP; defined as inability to sit unsupported [children > 6 months] or inability to suck [children ≤ 6 months]); and absence of any other obvious cause of severe illness[15] (Table 1).

Genotypes at three nucleotide sites, 202, 376, and 968, were determined using one of several standard methods to assess genotype differences between mild and severe malaria. We performed Sequenom analyses for all sites (primers in Table 2). Additionally, for the T968C polymorphism, we amplified the surrounding region (forward primer: CATC TGTGGCCACAGTCATC; reverse primer: TGGTCATCA TCTTGGTGTAC, T annealing = 57°C), digested the amplified fragments using the MspI restriction enzyme (New England Biolabs, Ipswich, MA) as described in the work by Rodrigues and others,[16] and confirmed that it remains a valid genotyping approach in resource-limited settings. Amplification reactions were performed with Taq Platinum according to the manufacturer's protocols (Invitrogen/Thermo Fisher Scientific, Waltham, MA). T968C was further genotyped through Sanger sequencing using the same primers and the same polymerase chain reaction (PCR) protocol. For the few samples in which genotypes were not completely clear as well as some random samples (for a total of 47), PCR fragments were run on a 1% agarose gel and isolated using the Wizard SV Gel and PCR Clean-Up System (Promega, Madison, WI), according to the manufacturer's protocol. A second nested PCR step (forward primer: ATCTGTGGC-CACAGTCATC; reverse primer: ACTGCTGGTGGAAGAT-GTC) was performed on isolated fragments, and Sanger sequencing was performed at the Vanderbilt University Genome Resources Core.

Although the 202 G->A mutation was too rare in Gambians (as expected) for us to draw any conclusion about protection (we only detected one female heterozygote and one male hemizygote with 202 G->A and mild malaria), 968 T->C yielded a highly significant result (Tables 3 and 4). The 202 G>-A alleles were only found in the presence of the T968 allele, which was expected. In the female severe malaria group, we observed a significant deficiency of 968 T->C heterozygotes and an excess of homozygotes (Table 3), producing a strong deviation from Hardy–Weinberg equilibrium (HWE; $P < 0.0001$) that remains significant after a Monte Carlo permutation test (10,000 runs, $P = 0.01$) using the HWSIM software (freely available at http://krunch.med.yale.edu/hwsim/). In contrast, in the mild malaria group, there was no significant deviation from HWE. The HWE test was not performed in the other variants, because we were not powered to detect any effects.

Consistent with the diagnosis, the severe malaria subjects had high parasitemia, with counts above 10^5 parasite/μL. We observed a significant difference in the distribution of 968 genotypes by malaria phenotypes when we combined hemizygous alleles with homozygous females (C with CC and T with TT; $P = 0.048$) (Table 5). Additional logistic regression analyses revealed that heterozygous females had odds ratios (ORs) significantly lower than one compared with either homozygous CC females or C males (OR = 0.06, $P = 0.030$) in unadjusted analyses (Table 6). Comparable results were obtained when adjusting for sex (OR = 0.06, $P = 0.027$) or sex and HbS genotype (OR = 0.08, $P = 0.047$) (Tables 7 and 8). Of note, genotype frequencies did not differ by ethnic groups; therefore, adjustment was not made for this variable (Table 9). Interestingly, Clark and others[14] described a protective effect for Gambian hemizygous males (1,294 severe malaria cases; OR = 0.77) and heterozygous females (1,194 severe malaria cases; OR = 0.71) but they did not detect a deviation from HWE for 968 T->C. For 202 G->A, they reported a significant P value (0.001), which however was not significant using the Monte Carlo permutation testing that we performed ($P = 0.07$)[14]; the permutation test would have been indicated in that study, because one genotypic class (i.e., 202 AA homozygotes) had an expected cell size of less than five.[14] Hence, their conclusion might not have been drawn had they used this analysis to assess protection.[14]

In conclusion, in the Gambian malaria sample that we studied, evidence for protection from severe disease was detected only in G6PD A- heterozygous females and not hemizygous males or homozygous females (phenotypically comparable with hemizygous males) based on both the heterozygote deficit that we observed among severe malaria cases and the logistic regression analyses. Although Bienzle and others[4] focused on G6PD deficiency and parasite counts rather than malaria disease and their sample was composed of subjects classified under a broad diagnosis of malaria, our data are most compatible with their proposed genotypic mode of protection. Nevertheless, the severe malaria phenotype is complex, and discrepancies between studies may be explained by differences in diagnostic criteria and underlying disease heterogeneity, which is clearly shown in Table 1; Table 1 highlights the clinical pleomorphism of severe malaria presentation in our Gambian sample. Therefore, genetic studies with *G6PD* (or other gene) variants associated with reduced morbidity and mortality and hence, conferring a selective advantage might miss a positive association signal if only selected clinical subsets and not others (such as SRD/hyperlactemia) are considered, thereby reducing the number of observed cases.

Additionally, the overall host–parasite dynamics are complex, and the protection in heterozygotes may be counterbalanced by a selective disadvantage for homozygous females, which was indicated by the excess of homozygotes with severe malaria, a result that deserves additional investigation.

TABLE 9
Distribution of genotypes 968 T->C among the ethnic groups

Genotype (%)	Mandingo ($N = 104$)	Wollof ($N = 32$)	Fula ($N = 56$)	Jola ($N = 41$)	Serere ($N = 12$)	Serahuli ($N = 7$)	Manjago ($N = 5$)	Aku ($N = 2$)	Unknown ($N = 22$)	P value
C or CC ($N = 7$)	4.8	0.0	1.8	0.0	0.0	0.0	0.0	0.0	4.5	0.722
C/T ($N = 11$)	2.9	3.1	1.8	9.8	8.3	0.0	20.0	0.0	0.0	0.260
T or TT ($N = 263$)	92.3	96.9	96.4	90.2	91.7	100.0	80.0	100.0	95.5	0.785

Information on ethnicity was available for 95.7% of all samples. For each of the main ethnic groups, the proportions of *G6PD* genotypes are shown. Homogeneity of the frequencies of *G6PD* genotypes across different ethnic groups was assessed by χ^2 test.

We acknowledge that an important limitation of our analysis is the small sample size, especially in males. Therefore, additional studies of larger samples will be required to confirm the effect described in this study. Nonetheless, our results support a previously identified protective association and are in contrast to the alternative. Clearly, careful clinical characterization will need to be a major part of any study of this genetic effect.

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