Epistasis between the haptoglobin common variant and α+thalassemia influences risk of severe malaria in Kenyan children

Short title: Haptoglobin and severe malaria

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Scientific category: Clinical Trials and Observations
Key points

- Epistasis between the haptoglobin common variants and α⁺thalassemia predicts the risk of severe malaria in Kenyan children.

- Epistasis may explain apparent differences in malaria association studies and varying gene frequencies for α⁺thalassemia and haptoglobin.
Abstract

Haptoglobin (Hp) scavenges free hemoglobin following malaria-induced hemolysis. Few studies have investigated the relationship between the common Hp variants and the risk of severe malaria, and their results are inconclusive. We conducted a case-control study of 996 children with severe *Plasmodium falciparum* malaria and 1220 community controls in Kilifi, Kenya and genotyped for Hp, HbS and α+thalassemia. HbAS and α+thalassemia homozygotes were protected from severe malaria (OR 0.12; 95% CI 0.07-0.18 and OR 0.69; 0.53-0.91 respectively). The risk of severe malaria also varied by Hp genotype, Hp2-1 was associated with greatest protection against severe malaria and Hp2-2 with greatest risk. We found a significant interaction between Hp genotype and α+thalassemia in predicting risk of severe malaria: Hp2-1 in combination with heterozygous or homozygous α+thalassemia was associated with protection from severe malaria (OR 0.73; 0.54-0.99 and OR 0.48; 0.32-0.73 respectively), but the combination of α+thalassemia and Hp2-2 was not protective (OR 1.10; 0.78-1.55). Meta-analysis of the current and published studies suggests that Hp2-2 is associated with an increased risk of severe malaria compared to Hp2-1 and that α+thalassemia modifies malaria risk. Epistasis between the common Hp variants and α+thalassemia could explain differing findings in malaria association studies and varying gene frequencies.
INTRODUCTION

Severe malaria ranks third among infectious causes of childhood death worldwide.\(^1\) Cell-free hemoglobin (Hb), released following intravascular hemolysis is highly toxic and may contribute to the pathogenesis of severe malaria by increasing inflammation, vasoconstriction and oxidant damage and by promoting adhesion of parasite-infected red blood cells to molecules such as ICAM-1.\(^2,3\) Haptoglobin (Hp) is an acute-phase plasma protein that binds rapidly and irreversibly with cell-free hemoglobin and the resultant Hp-Hb complexes are cleared via CD163 receptors on monocytes and tissue macrophages.\(^4,5\) Hp exists in three common variants in humans, the Hp1-1 dimer, the Hp2-1 linear polymer and the large Hp2-2 circular multimer.\(^4\) Hb binding to the various Hp proteins may vary, as seen in the Hp2-2-Hb complex, which has an unstable heme moiety and is itself redox active.\(^6\) Moreover, the Hp2-2 type is associated with Hb-mediated oxidant damage,\(^6-9\) inflammation,\(^10\) endothelial cell dysfunction\(^11,12\) and vasospasm following hemorrhage.\(^13,14\)

Whether the risk of severe malaria is altered by Hp type remains unclear. Four studies that have investigated this question have produced conflicting results.\(^15-18\) Early studies, conducted in Sudan and Ghana using plasma electrophoresis to determine Hp phenotype, suggested that the Hp1-1 type was associated with an increased risk of severe malaria.\(^15,16\) However, malaria-induced hemolysis depletes plasma Hp, and in the Ghanaian study 45% of children had an unknown or ‘Hp0’ phenotype\(^16\) while the prevalence of ‘Hp0’ was not reported in the Sudanese study.\(^15\) More recent studies, conducted in The Gambia and Northern Ghana using PCR-based genotyping methods, found no association between Hp genotype and severe malaria and an increased risk of severe malaria in children with the Hp2-2 genotype respectively.\(^17,18\) Recent studies
investigating the relationship between Hp genotype and the risk of uncomplicated malaria are similarly inconclusive, finding variously an increased risk of symptomatic malaria in Hp2-2 individuals, a reduced incidence among older children with the Hp2-2 genotype, and an increased placental parasite density among pregnant mothers with the Hp1-1 genotype. The effect of Hp concentration on the risk of severe malaria is also not known. Hp was toxic to malaria parasites in vitro and increased parasite burden was demonstrated in an Hp knock-out mouse. The -61C SNP in the Hp promoter region, which causes very low Hp concentrations, was associated with a reduced risk of uncomplicated malaria.

Since the common HP allele arises from a large gene duplication and cannot be uniquely identified by a SNP, it has not been included in recent genome-wide association studies of severe malaria. Here we report the results of the largest case-control study conducted to date (n=996 severe malaria cases and 1220 controls) that has investigated the association between Hp genotype and susceptibility to severe P. falciparum malaria. We further considered for the first time possible interactions between Hp and two other malaria resistance genes, HbAS and α-thalassemia.
METHODS

Study population

The study was conducted in Kilifi district on the coast of Kenya, where the majority of the population are rural dwellers of the Mijikenda ethno-linguistic group. The clinical characteristics and epidemiology of malaria in the study area have been described in detail previously.\(^\text{30,31}\) Case patients were children (\(<14\) yrs of age) who were residents of the study area served by the Kilifi Health and Demographic Surveillance System\(^\text{32}\) and who were admitted to the High Dependency Unit at Kilifi District Hospital between 7\(^{th}\) January 2001 and 6\(^{th}\) January 2010 with severe malaria. Severe malaria was defined as a positive blood film for \textit{P. falciparum} parasites in association with one or more of the following: prostration (Blantyre Coma Score (BCS) 3-4), coma (BCS \(\leq 2\)), or respiratory distress (intercostal recession or deep or laboured breathing).\(^\text{30}\) Children with uncomplicated severe malarial anemia (i.e. Hb \(<5\text{g/dl}, \text{but with no signs of respiratory distress, prostration or any other complications})\) are not routinely admitted to our High Dependency Unit and were not included in our study, since uncomplicated severe malarial anemia (SMA), when treated with transfusion, does not require intensive supportive care and is associated with a mortality of \(<1\%\).\(^\text{30}\) All case patients were treated according to standard guidelines as described in detail previously.\(^\text{33}\) The collection of case samples is summarized in Figure 1.

Controls were children born within the same study area as cases between August 14\(^{th}\) 2006 and August 20\(^{th}\) 2010, and were sampled as part of a cohort study of genetic susceptibility to infectious diseases during home visits at 3-11 months of age.\(^\text{34}\) Individual written informed consent was provided by all study participants or their
parents/guardians. The study was approved by the KEMRI/National Ethical Review Committee in Nairobi. The study was conducted in accordance with the Declaration of Helsinki.

**Laboratory methods**

Routine hematologic, biochemical and malaria parasite data were collected for all case children using standard methods as described previously.\(^3\) DNA was extracted by standard methods using proprietary kits (Qiagen DNA Blood Mini Kit; Qiagen, West Sussex, United Kingdom; or Puregene; Gentra Systems, Minneapolis, MN) and Hp genotypes determined by allele-specific PCR as previously described.\(^3\) Hemoglobin typing for HbAA, HbAS and HbSS was conducted by either electrophoresis using cellulose acetate gels (Helena) or by HPLC (Variant analyser, BioRad, Hercules, CA, USA) using the β-thalassemia short program while the α\(^-3.7\) deletion in the α-globin gene, the common form of α\(^+\)thalassemia in Africa, was typed by PCR as described previously.\(^3\) The Hp promoter polymorphism, A-61C (rs 5471), was typed at the Wellcome Trust Centre for Human Genetics, Oxford, UK by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry using the SEQUENOM\(^\circledR\) system (San Diego, CA, USA).\(^3\)

**Statistical analyses**

All analyses were conducted using Stata V12.0 (StataCorp. College Station, TX, USA). Non-normally distributed variables were log-transformed prior to analysis. Categorical data were analysed using the χ\(^2\) test, while the distribution of continuous variables by Hp genotype was investigated using linear regression. The association between Hp genotype and severe malaria was determined by ANOVA. Logistic
regression was used to derive odds ratios (ORs) for risk of severe malaria overall, severe malaria subgroups, and in-patient death according to Hp genotype in univariable and multivariable models adjusted for potential confounders including gender, ethnicity, Hb type (HbAS) and α-thalassemia genotype. Interactions between Hp genotype and other explanatory variables were assessed using the likelihood-ratio test.

**Meta-analysis of studies of Hp type and severe malaria**

The following criteria were applied to potential studies for inclusion in the meta-analysis: (i) indexed in PubMed (NCBI) under the search terms ‘severe malaria’ and ‘haptoglobin phenotype’ or ‘haptoglobin genotype’ (ii) original studies in which both case patients and controls were children; and (iii) a case-control study design with clear comparative groups of cases with severe malaria and controls without severe malaria. Four studies were identified\(^{15-18}\) of which two were excluded: one because the study also included adult cases and a control group that consisted only of adults recruited among the staff, technicians, workers and students at the institute\(^{15}\) and a second because 45% of cases and controls were of unknown (Hp0) phenotype.\(^{16}\) Data from the two remaining studies\(^ {17,18}\) were extracted and summarized and meta-analysis was performed using STATA V.12.0.
RESULTS

Characteristics of severe malaria patients

Our study included 996 case patients with severe malaria and 1220 controls. The median age of patients was 27.2 months (range, 0-147), 49.8% were male, and 97.2% were of the Mijikenda ethnic group. The characteristics of case patients, stratified by Hp genotype, are summarised in Table 1. Overall, the geometric mean \(P. falciparum\) parasite density and hemoglobin concentrations were 57,435/μl (95% CI 49,405-66,770) and 6.6 g/dL (95% CI 6.5-6.8) respectively while the proportions of severe malaria patients with HbAS, \(-α/α\) thalassemia, \(-α/-α\) thalassemia and Hp-61C were 2.2%, 48.3%, 11.8% and 20.2% respectively. The demographic characteristics of severe malaria patients did not vary according to Hp genotype (Table 1). Cerebral malaria was diagnosed in 528 children (53.1%), severe anemia in 224 children (22.6%), and 104 children (10.5%) had both cerebral malaria and severe anemia. Other non-exclusive categorizations of severe malaria, stratified by Hp genotype, are summarised in Table 2. Ninety-four severe malaria patients (9.4%) died.

Haptoglobin genotype and risk of severe malaria

Hp genotype was associated with risk of severe malaria by ANOVA (P=0.01 before and after adjustment for gender, ethnicity, Hb type (HbAS) and \(α^{+}\)thalassemia genotype). We therefore used logistic regression models to determine which of the three two-way comparisons between Hp genotypes accounted for this statistically significant variation in risk (Table 3).

Hp2-2 was associated with greatest risk and Hp2-1 with least risk of severe malaria. The risk of severe malaria did not differ significantly between Hp1-1 and Hp2-2.
genotypes (P=0.32) and varied with marginal significance between Hp1-1 and Hp2-1 genotypes (P=0.06, Table 3). However, the risk of severe malaria was significantly increased in the Hp2-2 genotype when compared to the Hp2-1 genotype (OR 1.35; 1.10-1.67; P=0.005) or when compared to Hp2-1 and Hp1-1 genotypes combined (OR 1.26; 1.03-1.53; P=0.02). Conversely, the Hp2-1 genotype was associated with 26% protection against severe malaria when compared to Hp2-2 (OR 0.74; 0.60-0.91; P=0.005) or 21% protection when compared to the Hp2-2 and Hp1-1 genotypes combined (OR 0.79; 0.67-0.93; P=0.006) indicating a heterozygote advantage.

Odds ratios were significantly raised in the Hp2-2 genotype compared to the Hp2-1 genotype for all categories of severe malaria except for symptomatic SMA in which there was a non-significantly increased risk (P=0.10, Table 3). Hp2-2 was also associated with a marginally significant increased OR for death compared to Hp1-1. Compared to children of the Hp2-1 genotype, those of the Hp1-1 genotype were at significantly increased risk of deep breathing and metabolic acidosis (Table 3). Parasite densities did not differ significantly between the Hp genotypes.

**Hp A-61C promoter polymorphism**

In order to investigate the effect of Hp concentration on the risk of severe malaria we typed the Hp A-61C promoter SNP, which is known to be associated with very low Hp concentrations,25 in a subset of 519 case patients and 876 controls for whom DNA samples were available in the UK. The characteristics of the subset typed did not differ from the non-typed group in any respect except with regard to subgroups within the Mijikenda ethnic group (the typed subset included more of the Kambe group and the non-typed group more of the Kauma group, P<0.05). The -61C SNP, present in
104 severe malaria cases (20.2%) and in 167 controls (19.1%), was significantly associated with the HP\(^2\) allele as previously reported\(^{21}\) and no -61CC homozygotes were identified. We found no association between the -61C allele and the risk of severe malaria (OR 1.05; 0.80-1.38; P=0.75 unadjusted and OR 0.91; 0.54-1.52; P=0.71 adjusted) or any evidence for an interaction between the -61C SNP and the common Hp variants in predicting risk of severe malaria (P=0.66 in likelihood-ratio testing).

HbS

Hb typing was successful in 990 cases (99%) and 1220 controls (100%). HbAS, carried by 22 cases (2.2%) and 200 controls (16.4%), was associated with 88% protection against severe malaria (OR 0.12; 0.07-0.18; P<0.0005). We found no evidence for an interaction between Hp genotype and hemoglobin type (HbAA, HbAS) in predicting the risk of severe malaria (P=0.28 in likelihood-ratio testing), however given the small number of children with HbAS among our severe malaria cases (n=22/990) our study was underpowered to find such an effect.

\(\alpha^+\) thalassemia

The heterozygous \(\alpha^+\)thalassemia (-/\alpha\alpha) was carried by 481 cases (48.3%) and 591 controls (48.4%) while homozygous \(\alpha^+\)thalassemia (-/\alpha-\alpha) was carried by 117 cases (11.8%) and 187 controls (15.3%). The -/\alpha-\alpha genotype was associated with 31% protection against severe malaria (OR 0.69; 0.53-0.91; P=0.008) while the -/\alpha/\alpha thalassemia type was not significantly associated with protection (P=0.27). However, we found statistically significant evidence for an interaction between \(\alpha^+\)thalassemia and Hp type in predicting the risk of severe malaria (P=0.02 in likelihood-ratio testing).
testing). The risk of severe malaria was lowest for \( \alpha/-\alpha \) thalassemia inherited in combination with Hp2-1, but \( \alpha^+/\alpha \) thalassemia was not protective when inherited in combination with Hp2-2. Hp1-1 was associated with a non-significant trend towards protection against severe malaria when inherited with \( \alpha^+ \) thalassemia (Figure 2). Odds ratios for the risk of severe malaria stratified by \( \alpha^+ \) thalassemia are shown in Table 4 illustrating the effect of \( \alpha^+ \) thalassemia in modifying risk associated with Hp genotype.

**Meta-analysis**

In addition to our current study we identified two eligible additional studies relating to Hp type and the risk of severe malaria\(^{17,18} \) for inclusion in a meta-analysis. Our analysis suggested that the Hp2-2 genotype is associated with an increased risk of severe malaria compared to the Hp2-1 genotype (OR=1.20; 95% CI 1.02-1.42; \( P=0.028 \), Figure 3), but that there is no significant variation in risk of severe malaria between Hp2-2 and Hp1-1 or between Hp1-1 and Hp2-1. We further stratified the current study by \( \alpha^+ \) thalassemia and showed significant heterogeneity between these groups in predicting risk of severe malaria for Hp2-2 vs. Hp2-1 (\( P=0.006 \)); the odds ratio for severe malaria was 0.93 (0.67-1.30) in the group without \( \alpha^+ \) thalassemia and 1.71 (1.30-2.24) in the group with \( \alpha^+ \) thalassemia (Figure 3).
DISCUSSION

In a large case-control study of 996 severe malaria case children and 1220 controls we found that in Kilifi, Kenya, the risk of severe malaria is clearly associated with the common Hp variants. The highest risk for severe malaria was found in the Hp2-2 genotype and the lowest risk in the Hp2-1 genotype. This finding was supported by a meta-analysis of this and two earlier studies that suggested an increased risk of severe malaria in Hp2-2 compared to Hp2-1 individuals. Hp1-1 individuals may have had an increased risk of severe malaria compared to Hp2-1 individuals, but this was not supported on meta-analysis. In sub-group analyses, we found that the increased risk of severe malaria among Hp2-2 subjects was seen in all the severe malaria syndromes although this did not reach statistical significance in the symptomatic SMA group. Finally, we found evidence to suggest that the effect of Hp on the risk of severe malaria is modified by α+thalassemia.

Our finding of an increased risk of severe malaria among Hp2-2 individuals is in apparent contradiction to two earlier studies conducted in Sudan and Ghana,15,16 both of which reported a reduced risk of severe malaria among children of the Hp2-2 phenotype. This might be explained by the fact that in these previous studies Hp type was based on phenotyping rather than genotyping, since Hp2-2 is present at lower plasma concentrations and is more likely to be depleted during malaria-related hemolysis and therefore lead to biased misclassification. Similarly, our findings are not in complete agreement with two studies conducted by genotyping. In one study, conducted in Northern Ghana, the authors found evidence for a marginally increased risk of severe malaria in Hp2-2 individuals18 while in a second study, conducted in The Gambia, the authors found no association between Hp genotype and severe
malaria risk.\textsuperscript{17} It is possible that the clinical characteristics of case patients included in these studies may have differed from those included in our current study. In our study we did not include children presenting with uncomplicated severe malaria anemia since this condition is not associated with significant mortality.\textsuperscript{30} Differences in ethnicity and genetic diversity may be another explanation. Finally, the varying observations made in different populations might also be explained by epistasis, which holds that the effect of a genotype at a particular locus depends on the genotype co-inherited at a second unrelated locus. Epistasis is likely to be more common than previously thought.\textsuperscript{38}

We looked for possible epistasis between Hp genotype and two known malaria-resistance genes, HbAS, which results from a structural abnormality of the $\beta$-globin chains of hemoglobin, and $\alpha^+$thalassemia which results from the underproduction of normal $\alpha$-globin chains of hemoglobin. The number of children with HbAS and severe malaria were few ($n=22$) and our study was underpowered to investigate any potential epistasis between HbAS and Hp genotype. However, we did find evidence for an epistatic interaction between $\alpha^+$thalassemia and Hp genotype ($P=0.02$). Our data suggest that $\alpha^+$thalassemia inherited in combination with Hp2-1 is strongly protective against severe malaria (37% protection, $P=0.003$), but that protection is considerably reduced when inherited in combination with Hp1-1 (13% protection, $P=0.39$), and is lost altogether when inherited in combination with Hp2-2 (Table 4). Similarly, although Hp genotype inherited in combination with $\alpha^+$thalassemia significantly altered the risk of severe malaria Hp genotype in the absence of $\alpha^+$thalassemia did not appear to alter risk in sub-group analyses (Table 4). While this possible epistasis should be viewed with caution, it seems plausible that varying
frequencies of α⁺thalassemia across Africa might explain apparent differences in the results of previous studies investigating the association between Hp type and severe malaria. For example, the frequency of the α⁺3.7 allele, the most common cause of α⁺thalassemia in sub-Saharan Africa, is 0.12 in The Gambia, 0.33 in Ghana and 0.40 in our population. Varying Hp² allele frequencies could similarly explain differing findings for α⁺thalassemia association studies.

If epistasis exists between the common Hp variants and α⁺thalassemia, by what mechanism might it act? Despite high population frequencies of α⁺thalassemia and the Hp² allele in many malaria-endemic areas, little is known about the pathophysiological consequences of their co-inheritance. A number of mechanisms for an epistatic interaction seem plausible, a few of which are summarized here.

Individuals with α⁺thalassemia are under increased oxidative stress, a fact that likely relates to an intra-erythrocytic excess of unmatched β chains and reactive free thiols. Hp2-2, unlike Hp1-1 and Hp2-1, is significantly less able to quench hemoglobin-iron mediated oxidant stress, which may account for reduced ferroxidase and vitamin C levels in Hp2-2 individuals. Additionally, methemoglobin, an endothelial-cell activator, is increased in thalassemia and may be further increased in Hp2-2 individuals following hemolysis due to an oxidatively unstable heme moiety in the Hp2-2-Hb complex. The Hp2-2 variant is further associated with a shift towards a pro-inflammatory Th1 cytokine response. Thus, it is possible that the protection conferred by α⁺thalassemia is lost when co-inherited with Hp2-2 due to excessive oxidant damage, inflammation and endothelial cell activation, especially within the brain and deep tissues where the large Hp2-2 protein is less able to penetrate. Indeed, subarachnoid hemorrhage in patients with the Hp2-2 genotype results in more
severe vasospasm, tissue ischemia and inflammation.\textsuperscript{13,14,48} Additionally, the reduced rosetting\textsuperscript{49-51} and endothelial cell adherence\textsuperscript{51} observed in $\alpha^+\text{thalassemia}$ may be countered by the up-regulation of vascular adhesion molecules such as VCAM and endothelial cell dysfunction observed in Hp2-2 individuals\textsuperscript{11,12} resulting in loss of protection in individuals co-inheriting both genotypes.

Conversely, Hp2-1 co-inherited with $\alpha^+\text{thalassemia}$ appeared to confer significant protection from severe malaria indicating heterozygote advantage. In addition to reduced rosetting, infected $\alpha^+\text{thalassemia}$ cell membranes bind significantly more malaria immune globin and are more susceptible to phagocytosis compared to control cells,\textsuperscript{52,53} a process likely mediated by oxidative damage to the red cell membrane from unbound $\beta$ chains and accelerated acquisition of aggregated band 3 proteins.\textsuperscript{53,54}

It is possible that the Hp2-1 variant optimizes the protective effect of $\alpha^+\text{thalassemia}$ by providing a balanced plasma environment with optimal oxidant/anti-oxidant levels and Th1 vs. Th2 cytokines compared to Hp1-1 and Hp2-2. Hp2-1 has a distinct molecular structure compared to the other variants, for example $V.\text{vulnificus}$ is unable to acquire iron from Hp2-1-Hb complexes, despite iron acquisition from Hp1-1-Hb and Hp2-2-Hb complexes.\textsuperscript{55} Heterozygote advantage from Hp2-1 is further observed with protection from Kaposi’s sarcoma\textsuperscript{56} and reduced mortality in HIV patients.\textsuperscript{57} A similar trend towards protection was seen in individuals co-inheriting Hp1-1 and $\alpha^+\text{thalassemia}$ although this did not reach statistical significance. Epistasis may provide valuable insights into the mechanisms by which malaria resistance genes provide protection.
We did not find an interaction between the common Hp variants and the -61C allele or any influence of the -61C allele on the risk of severe malaria. It might be expected that low Hp concentrations associated with the -61C allele\textsuperscript{25,26} would expose children to the deleterious effects of cell-free Hb and therefore predispose such children to severe malaria.\textsuperscript{2} However, in our previous study of children with uncomplicated malaria, although the -61C allele was associated with reduced Hp concentrations during convalescence when malaria parasites had been cleared, it had no influence on Hp concentration at the time of a malaria episode.\textsuperscript{21} Hp is up-regulated during the acute phase response and this may counter the reduced transcription that might result from the -61C allele outside the acute phase response. Alternatively, it is also possible that the structure or function of the common Hp variants may be more clinically relevant than their concentrations. Our finding differs from that made in our earlier study conducted in The Gambia, in which a reduced incidence of uncomplicated malaria was found only in children >3 years of age carrying the -61C allele.\textsuperscript{21} Since this protection was only seen in older children, we proposed that an immune mediated mechanism, possibly mediated by low Hp concentration, oxidative damage to the red cell membrane and accelerated acquisition of aggregated band 3 proteins or by other immune modulation might have been involved.\textsuperscript{21} In the current study the majority of children were <3 years of age and as such will have had less time to acquire immunity. The patient mix was also different between the studies. Children in the current study had severe life-threatening malaria requiring high dependency care in hospital while the previous study involved children in the community with a mild febrile episode associated with malaria parasites on blood film. Moreover, although Hp concentration at convalescence might influence acquisition of immunity following uncomplicated malaria, Hp concentration at the time of malaria may be more
critically important in severe malaria. It also seems possible that the apparently conflicting findings between these two studies could be explained by a range of other genetic, ethnic or environmental differences between the children involved. We also previously reported a lower incidence of uncomplicated clinical malaria in older children (>4 years) carrying the Hp2-2 genotype, postulating that this might be mediated by oxidative damage to the red cell membrane and accelerated acquisition of antibodies to aggregated band 3 proteins. Similarly, in the current study, the majority of children were <4 years old and thus would have had less time to acquire immunity.

In summary, this large case control study and meta-analysis suggest that the common Hp 2-2 genotype confers an increased risk of severe malaria and that the Hp2-1 genotype is protective. Moreover, our data are compatible with an epistatic interaction with regard to susceptibility to severe malaria between Hp and α+thalassemia. It seems plausible that heterozygote advantage, balancing selection pressure and epistasis might explain the varying gene frequencies of the \( HP^2 \) allele and \( \alpha^+ \)thalassemia in different populations and explain why the relationship between malaria and \( HP^2 \) has proved difficult to confirm. Further studies investigating these associations in other malaria-endemic populations are indicated.
Acknowledgements

We thank the study participants, their families and the nurses and fieldworkers who participated in this study and Kevin Marsh, Kathryn Maitland and Charles Newton for their contribution to the study. We thank David Weatherall and Andrew Prentice for helpful discussions relating to $\alpha^+$thalassemia and haptoglobin. We thank the staff of the human genetics laboratory at the KEMRI-Wellcome Trust Programme including Herbert Opi, Metrine Tendwa, Johnstone Makale, Adan Mohamed, Kenneth Magua, and Ruth Mwarabu for their help with sample processing, genotyping and database support. TNW is funded by a fellowship from the Wellcome Trust (091758) and by funds from the European Union FP7 EVIMalR Consortium. SHA was supported by a Career Support Award from Oxford University Clinical Academic Graduate School and awards from the European Society of Pediatric Infectious Disease (ESPID), The Academy of Medical Sciences, The Wellcome Trust, The British Heart Foundation and Arthritis Research UK. This paper is published with permission from the Director of KEMRI.

Authorship contributions

SHA conceived the study, performed experiments, analysed data and wrote the manuscript. SMU performed experiments, analysed data and contributed to writing the manuscript. EN and AWM performed experiments and contributed to data analysis. GN and CN managed databases and analysed data. DK conceived the study and performed experiments. KR performed experiments, contributed to data analysis and wrote the manuscript. TNW conceived the study, obtained funding, contributed to data analysis and wrote the manuscript. None of the authors have any conflicts of interest.
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Figure Legends

Figure 1. Study Construction

Between the 7th of January 2001 and the 6th of January 2010, 23,897 children were admitted to Kilifi District Hospital from the study area served by the Kilifi Health and Demographic Surveillance System (DSS) and of these 3271 were admitted to the Children’s High Dependency Unit (HDU). Severe life-threatening malaria was diagnosed in 1127 patients on the HDU. Eighty-two samples were missing or had insufficient DNA for genotyping, 23 samples could not be typed for Hp, 16 samples could not be typed for α-thalassemia and 10 samples were missing clinical data. A total of 996 severe malaria cases had complete genotyping and clinical data and were included in the study.

Figure 2. Odds ratios for severe malaria by hemoglobin (HbAS), α+thalassemia and haptoglobin genotypes

Logistic regression models were used to determine odds ratios for risk of severe malaria by (A) Hemoglobin type (HbAS), α+thalassemia type and Hp genotype. HbAA, αα/αα and Hp1-1 were the respective reference types with an odds ratio for severe malaria of 1.0. (B) Hemoglobin type (HbAS) and α+thalassemia type stratified by Hp genotype. HbAA and Hp1-1 and αα/αα and Hp1-1 were the respective reference types with an odds ratio for severe malaria of 1.0.

Figure 3. Meta-analysis of studies examining the relationship between haptoglobin type and severe malaria

Forest plots showing a meta-analysis of eligible case-control studies that have examined the relationship between Hp type and the risk of severe malaria. Individual
estimates and the relative contribution of the individual studies to the overall estimates (% weight and numbers in each group) are shown. *The frequency of the –\(\alpha/\) allele in the relevant country. The studies by Aucan et al\textsuperscript{17} and Bienzle et al\textsuperscript{18} do not report the prevalence of \(\alpha^+\) thalassemia in their study populations and the prevalences shown (0.12 in The Gambia\textsuperscript{39} and 0.33 in Ghana\textsuperscript{40}) are from other studies within the same country. Further Forest plots show the results of the current study stratified by \(\alpha^+\) thalassemia status.
Table 1. Characteristics of severe malaria patients by haptoglobin genotype

<table>
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<th>Characteristic</th>
<th>Hp 1-1</th>
<th>Hp 2-1</th>
<th>Hp 2-2</th>
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<td>No. (%)</td>
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<td>Median age, mo (IQR)</td>
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<td>Girama</td>
<td>168 (53.9)</td>
<td>225 (53.2)</td>
<td>157 (60.2)</td>
</tr>
<tr>
<td>Chonyi</td>
<td>82 (26.3)</td>
<td>119 (28.1)</td>
<td>56 (21.5)</td>
</tr>
<tr>
<td>Kauma</td>
<td>35 (11.2)</td>
<td>41 (9.7)</td>
<td>21 (8.1)</td>
</tr>
<tr>
<td>Other Mijikenda</td>
<td>19 (6.1)</td>
<td>21 (5.0)</td>
<td>24 (9.2)</td>
</tr>
<tr>
<td>Non-Mijikenda</td>
<td>8 (2.6)</td>
<td>17 (4.0)</td>
<td>3 (1.2)</td>
</tr>
<tr>
<td>HbS type no. (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hb AA</td>
<td>300 (96.5)</td>
<td>408 (97.6)</td>
<td>254 (97.3)</td>
</tr>
<tr>
<td>Hb AS</td>
<td>10 (3.2)</td>
<td>7 (1.7)</td>
<td>5 (1.9)</td>
</tr>
<tr>
<td>Hb SS</td>
<td>1 (0.3)</td>
<td>3 (0.7)</td>
<td>2 (0.8)</td>
</tr>
<tr>
<td>α^+ thalassemia genotype no. (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>αα/αα</td>
<td>126 (40.4)</td>
<td>170 (40.2)</td>
<td>102 (39.1)</td>
</tr>
<tr>
<td>α-/αα</td>
<td>150 (48.1)</td>
<td>203 (48.0)</td>
<td>128 (49.0)</td>
</tr>
<tr>
<td>α^-/α</td>
<td>36 (11.5)</td>
<td>50 (11.8)</td>
<td>31 (11.9)</td>
</tr>
<tr>
<td>Body mass index (n=893)^a</td>
<td>10.6 (10.2-11.0)</td>
<td>10.2 (9.9-10.6)</td>
<td>10.3 (9.9-10.7)</td>
</tr>
<tr>
<td>Parasitemia, log_{10} value, µL (n=988)</td>
<td>4.58 (4.45-4.71)</td>
<td>4.64 (4.53-4.75)</td>
<td>4.67 (4.54-4.81)</td>
</tr>
<tr>
<td>Hemoglobin, g/dL (n=993)</td>
<td>6.6 (6.3-6.9)</td>
<td>6.6 (6.4-6.8)</td>
<td>6.7 (6.4-7.0)</td>
</tr>
</tbody>
</table>

Numbers (with percentages) are presented for categorical variables. Geometric means are presented with 95% confidence intervals for continuous variables.

^a Due to severity of illness a proportion of children did not have height measured.
Table 2. Haptoglobin gene frequencies in controls and severe malaria cases

<table>
<thead>
<tr>
<th></th>
<th>All genotypes</th>
<th>Hp 1-1</th>
<th>Hp 2-1</th>
<th>Hp 2-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>1,220</td>
<td>361 (29.6)</td>
<td>590 (48.4)</td>
<td>269 (22.0)</td>
</tr>
<tr>
<td>Case patients with severe malaria</td>
<td>996</td>
<td>312 (31.3)</td>
<td>423 (42.5)</td>
<td>261 (26.2)</td>
</tr>
<tr>
<td>Severe malaria syndromes</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cerebral malaria, coma</td>
<td>528/995 (53.1)</td>
<td>172 (32.6)</td>
<td>224 (42.4)</td>
<td>132 (25.0)</td>
</tr>
<tr>
<td>Prostration†</td>
<td>237/992 (23.9)</td>
<td>75 (31.7)</td>
<td>97 (40.9)</td>
<td>65 (27.4)</td>
</tr>
<tr>
<td>Deep breathing‡</td>
<td>412/995 (41.4)</td>
<td>137 (33.3)</td>
<td>172 (41.7)</td>
<td>103 (25.0)</td>
</tr>
<tr>
<td>Coma and deep breathing</td>
<td>211/995 (21.2)</td>
<td>68 (32.2)</td>
<td>86 (40.8)</td>
<td>57 (27.0)</td>
</tr>
<tr>
<td>Metabolic acidosis§</td>
<td>542/891 (60.8)</td>
<td>186 (34.3)</td>
<td>216 (39.9)</td>
<td>140 (25.8)</td>
</tr>
<tr>
<td>Symptomatic SMA¶</td>
<td>224/993 (22.6)</td>
<td>70 (31.3)</td>
<td>96 (42.9)</td>
<td>58 (25.9)</td>
</tr>
<tr>
<td>Fatal outcomeǁ</td>
<td>94/996 (9.4)</td>
<td>20 (21.3)</td>
<td>47 (50.0)</td>
<td>27 (28.7)</td>
</tr>
</tbody>
</table>

Because children may manifest multiple complications of severe malaria, some subjects contributed data to more than one clinical subgroup. Figures are number (and percentage) of group manifesting clinical characteristic. *Defined as Blantyre coma score of ≤2. †Defined as Blantyre coma score of 3-4. ‡Defined as previously described. §Defined as a base deficit >8 mM. ¶Hemoglobin less than 5 g/dl, falciparum malaria and one or more complications of coma, prostration, deep breathing or acidosis. ‖Deaths in hospital after admission.
<table>
<thead>
<tr>
<th></th>
<th>Hp1-1 vs. Hp2-1</th>
<th>Hp 1-1 vs. Hp2-2</th>
<th>Hp2-2 vs. Hp2-1</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>OR (95% CI)</td>
<td>P</td>
<td>OR (95% CI)</td>
</tr>
<tr>
<td>All severe malaria</td>
<td>1.21 (0.99-1.47)</td>
<td>0.06</td>
<td>0.89 (0.71-1.12)</td>
</tr>
<tr>
<td>All severe malaria adjusted</td>
<td>1.24 (1.01-1.53)</td>
<td>0.04</td>
<td>0.92 (0.72-1.16)</td>
</tr>
<tr>
<td>Severe malaria syndromes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Coma</td>
<td>1.30 (0.99-1.71)</td>
<td>0.06</td>
<td>1.01 (0.76-1.36)</td>
</tr>
<tr>
<td>Prostration</td>
<td>1.35 (0.96-1.89)</td>
<td>0.09</td>
<td>0.90 (0.61-1.33)</td>
</tr>
<tr>
<td>Deep breathing</td>
<td>1.35 (1.03-1.77)</td>
<td>0.03</td>
<td>1.0 (0.73-1.36)</td>
</tr>
<tr>
<td>Coma and deep breathing</td>
<td>1.38 (0.97-1.97)</td>
<td>0.08</td>
<td>0.91 (0.61-1.35)</td>
</tr>
<tr>
<td>Metabolic acidosis</td>
<td>1.46 (1.14-1.90)</td>
<td>0.003</td>
<td>1.03 (0.76-1.36)</td>
</tr>
<tr>
<td>Symptomatic SMA</td>
<td>1.22 (0.87-1.72)</td>
<td>0.25</td>
<td>0.90 (0.61-1.33)</td>
</tr>
<tr>
<td>Fatal outcome</td>
<td>0.71 (0.41-1.23)</td>
<td>0.22</td>
<td>0.54 (0.30-1.0)</td>
</tr>
</tbody>
</table>

Because children may manifest multiple complications of severe malaria, some subjects contributed data to more than 1 clinical subgroup. *Compared with Hp2-1 genotype as reference group, analysed in a logistic regression model including all genotypes; †Compared with Hp2-2 genotype as reference group, analysed in a logistic regression model including all genotypes. ‡Adjusted for gender, ethnic group, sickle status and α-thalassemia type. §Defined as in Table 2.
Table 4. Odds ratios for severe malaria by haptoglobin genotype stratified by α⁺ thalassemia genotype

<table>
<thead>
<tr>
<th>α⁺ thalassemia</th>
<th>Haptoglobin</th>
<th>Severe malaria (n)</th>
<th>Controls (n)</th>
<th>Odds ratio (95% CI)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>α⁺/α⁺</td>
<td>Hp1-1</td>
<td>126</td>
<td>133</td>
<td>1.0 (Reference)</td>
<td>Reference</td>
</tr>
<tr>
<td></td>
<td>Hp2-1</td>
<td>170</td>
<td>188</td>
<td>0.93 (0.67-1.30)</td>
<td>0.70</td>
</tr>
<tr>
<td></td>
<td>Hp2-2</td>
<td>102</td>
<td>121</td>
<td>0.89 (0.61-1.30)</td>
<td>0.55</td>
</tr>
<tr>
<td>-α/α⁺ and -α/-α</td>
<td>Hp1-1</td>
<td>186</td>
<td>228</td>
<td>0.87 (0.63-1.20)</td>
<td>0.39</td>
</tr>
<tr>
<td></td>
<td>Hp2-1</td>
<td>253</td>
<td>402</td>
<td>0.63 (0.47-0.86)</td>
<td>0.003</td>
</tr>
<tr>
<td></td>
<td>Hp2-2</td>
<td>159</td>
<td>148</td>
<td>1.10 (0.78-1.55)</td>
<td>0.60</td>
</tr>
</tbody>
</table>

Odds ratios were derived by logistic regression analyses adjusted for gender, ethnicity and sickle status. Hp1-1 inherited with α⁺/α⁺ was the reference type with an odds ratio for severe malaria of 1.0.
Figure 1.

23,897 children (aged <14 years) admitted to Kilifi District Hospital from DSS study area from 7th Jan 2001 to 6th Jan 2010

3,271 children transferred to Children’s High Dependency Unit

1,127 children diagnosed with severe malaria on Children’s High Dependency Unit

- 82 insufficient or missing sample
- 23 failed typing for Hp
- 16 failed typing for α-thalassemia
- 10 missing clinical data

996 patients with severe malaria included in the study
Figure 2.
Epistasis between the haptoglobin common variant and α+ thalassemia influences risk of severe malaria in Kenyan children

Sarah H. Atkinson, Sophie M. Uyoga, Emily Nyatichi, Alex W. Macharia, Gideon Nyutu, Carolyne Ndila, Dominic P. Kwiatkowski, Kirk A. Rockett and Thomas N. Williams