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HAPTOGLOBIN LEVELS ARE ASSOCIATED WITH HAPTOGLOBIN GENOTYPE AND a^+-THALASSEMIA IN A MALARIA-ENDEMIC AREA

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Abstract. Haptoglobin (Hp) is an acute phase protein that removes free hemoglobin (Hb) released during hemolysis. Hp has also been shown to be toxic for malaria parasites. a^+-Thalassemia is a hemoglobinopathy that results in subclinical hemolytic anemia. a^+-Thassemia homozygosity confers protection against severe malarial disease by an as yet unidentified mechanism. Hp levels were measured in a serial cross-sectional survey of children in Madang Province, Papua New Guinea (PNG). Hp levels were related to age, Hp genotype, Hb levels, parasitemia, splenomegaly, and ot^+-thalassemia genotype. Surprisingly, children who were homozygous for a^+-thalassemia had significantly higher levels of Hp than did heterozygotes, after controlling for relevant confounders. We suggest that this is the result of either reduced mean cell Hb associated with a^+-thalassemia homozygosity or an elevated IL-6-dependent acute phase response.

Haptoglobin (Hp) is an acute phase protein with an important protective role in hemolytic disease. It binds irreversibly to hemoglobin (Hb) and reduces the oxidative and peroxidative potential of free Hb.[1] The Hp–Hb complex is endocytosed by macrophages through the acute phase-regulated and signal-inducing macrophage protein CD163.[2] Hp exists in three phenotypic forms, Hp1-1, 2-1, and 2-2, encoded by two co-dominant alleles, Hp^1 and Hp^2.[3] The Hp1 type may be further subdivided into two subtypes, Hp1S and Hp1F, coded by two alleles hp^{1F} and hp^{1S}.[4] Allele frequencies of types and subtypes vary according to geographical location.[4] The ability to bind Hb is phenotype-dependent and has been found to be in the order 1-1 > 2-1 > 2-2, the binding capacity reflecting the plasma levels of the three phenotypes.[5] Various factors play a role in determining Hp concentration (i.e., Hp phenotype, age, hemolysis, and the acute phase response).[6]

Hemolysis is a pathologic feature of malaria infection, and decreased Hp levels are associated with clinical malaria.[7] Hp levels are reduced by both chronic, low-density, asymptomatic parasitemia and possibly malaria-associated immune complex destruction of uninfected erythrocytes.[8] The prevalence of ahaptoglobinemia (absence of measurable Hp) is common in malaria endemic areas and increases with increasing malaria transmission.[9] Available data on the protective effect of Hp polymorphisms from West Africa have been geographically variable.[10–13]

Interestingly, Hp is directly toxic to *P. falciparum in vitro* at concentrations that may occur during an acute phase response.[14] The exact mechanism of action is not known. Hp does not enter the infected erythrocyte but may act indirectly, disrupting normal parasite protein trafficking within the host cell.[14] An *in vivo* model of malaria infection in mice has shown that parasite burdens and peak parasite densities were higher in Hp knockout mice.[15] Hp has other anti-microbial effects; Hp has been shown to make iron unavailable to bacteria using Hb (e.g., *E. coli*) and to cause agglutination of *Streptococcus pyogenes*.[16,17]

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Hemolysis is also a clinical feature of a^+-thalassemia, a non-structural inherited disorder of Hb. a^+-Thalassemia is a result of deletions (3.7 or 4.2 kb) or inactivation by a point mutation of one of the duplicated *a-globin* genes. The heterozygous and homozygous a^+-thalassemia genotypes are (-a/ota) and (-a/-a), respectively.[18] In general, those heterozygous and homozygous for a^+-thalassemia have lower levels of total Hb, mean cell Hb concentration, mean corpuscular volume, and mean cell Hb than non-thalassemics (aa/aa).[19] For all parameters, there is a greater difference between heterozygotes and homozygotes than heterozygotes and non-thalassemics.[18] Therefore, compared with non-thalassemics, heterozygotes do not present any clinical symptoms, whereas homozygotes are characterized by mild hypochromic microcytic anemia.[20,21] Individuals homozygous for a^+-thalassemia have also been shown to be protected against severe *P. falciparum* malaria as well as all cause admissions to hospital with other infections in Papua New Guinea (PNG).[22]

There have been a number of studies of Hp in malarious areas that have looked either at plasma levels or at Hp genotype but interestingly, not Hp genotype and levels in the same individuals.[8,23,24] More surprisingly, no one has examined the relationship between Hp levels and hemolytic disorders such as a^+-thalassemia in a malaria-endemic situation. Given that Hp has a potentially beneficial role in malaria infection because of its ability to abrogate damage caused by free Hb and the antiparasitic effect noted *in vitro*, we decided to look at the association between Hp levels, malariometric indices, and host genotype in a malaria-endemic population.

MATERIALS AND METHODS

Study site. The study was conducted in the Amele villages of Madang Province on the north coast of PNG where there is intense year-round transmission of malaria.[25] The region has a tropical climate with high annual rainfall (2.5-5 m) and high relative humidity (85-90%). A wet season is normally recognized between October and May. Temperatures vary little throughout the year (23°C average minimum to 30°C average maximum). The principal vectors of malaria in this area are members of the *Anopheles punctulatus* Dönitz complex. The mean number of infectious bites *(P. falciparum* and

P. vivax) per day in this region is estimated at 0.86.[26] Health services consist of an aid post providing basic health care, a health center at Yagaum and the nearest hospital is in Madang town, 35 km away by road.

Endemic malaria in the Amele is evidenced by decreasing parasite prevalence and splenic enlargement with age.[25] The spleen is enlarged during malaria because of removal of parasitized erythrocytes and hematopoiesis.[27] Rates of splenic enlargement and parasite rates decline substantially by the age of 15 years, indicating acquired immunity.[25] Young children less than 4 years of age have more frequent clinical episodes of malaria associated with high parasite densities.[28] Semi-immune children more than 4 years of age from Amele harbor chronic, asymptomatic malaria infections of *P. falciparum, P. vivax, P. malariae,* and *P. ovale,* with occasional episodes of clinical malaria associated with splenomegaly and parasitemia above the fever threshold of 1,000 parasites/ **(x**L of blood.[28],[29]

α^{+}-Thalassemia reaches very high frequencies in the Amele region.[30] The $-\alpha^{3.7}$ deletion can be of three different subtypes (I, II, III). $-\alpha^{3.7\ III}$ is only found in Melanesia and is the dominant form, except on the north coast of PNG, where $-\alpha^{4.2}$ predominates.[31] Polymorphisms such as Southeast Asian ovalocytosis (SAO) and glucose 6-phosphate dehydrogenase (G6PD) deficiency are also present in this area.[32],[33] The *Hp1* allele is present at a frequency of 69% in Melanesians, but the Hp1F form has not been reported.[34],[35]

Study design. A serial cross-sectional survey was conducted in asymptomatic children living in villages of the Amele region, Madang Province, PNG. The study took place from November to December of 1999 and 2000. The children were invited to attend the first cross-sectional study at school or by house visit after consultation with parents at a village meeting. The aim was to recruit 1,000 individuals 1-17 years of age in the first year and encourage them to return the following year.

One thousand fifteen individuals were recruited into the survey in 1999, and 623 individuals returned for the second data collection in 2000. A further 76 individuals were excluded because of insufficient or missing data. Loss to follow-up from the study took place predominantly in children 1-4 years of age who were surveyed at home. Disruption to parents' agricultural practice accounted for lack of compliance at the second survey. Those who had insufficient data were also in this age group because smaller children only had a finger prick of blood taken. The most reliable places sampled were schools, where children regularly attended lessons from 7 years of age.

Data collection. Axillary temperature was recorded for each child, and fever was defined as a temperature > 37.5°C. Examination for splenomegaly was conducted by detecting the number of finger widths of spleen detectable below the rib line (the Hacketts grading system). Venous blood was drawn using Vacutainer tubes (Becton Dickinson, Oxford, UK) containing EDTA as an anti-coagulant. A blood smear was immediately made and later stained with Giemsa. Levels of Hb were also immediately determined using a Hb photometer (HemoCue AB, Angelholm, Sweden). Malaria-related anemia was defined as Hb < 8 g/dL.[36] Whole blood was blotted onto Isocode Stix filter paper (Schleicher & Schuell, Dassel, Germany) following the manufacturer's instructions for further use in human genetic analyses. The remaining blood was centrifuged and separated into plasma (stored at -80°C), buffy coat and erythrocyte pellet, both stored in guanidine hydrochloride (G-HCl) at 4°C.

Parasitology. *Plasmodium* species and counts (number of parasites per 200 leukocytes) were analyzed by microscopy and recorded. A parasite negative slide was one on which 2,000 leukocytes were observed and no parasites seen. Duplicate readings were made for 20% of smears. Greater than 75% concordance in results was observed, and therefore initial results were accepted. This is the standard test of reproducibility carried on blood surveys by the PNG Institute of Medical Research. Generally, parasite counts are made per 200 leukocytes and converted assuming a standard leukocyte count of 8.0 **x** 10^3/(xL of blood. The most accurate counts of parasite density are obtained by counting parasites per leukocyte and converting this to parasites per microliter of blood using a mechanized Coulter leukocyte count of the same blood sample. Previous analysis has compared general parasite counts to parasite counts form Coulter counter leukocyte counts in asymptomatic children from Madang (M. Bruce, unpublished observations). Variation in the Coulter count in all age groups resulted in over-estimates of up to 2.0 times and under-estimates of up to 0.4 times the actual parasite density when a standard value of 8.0 **x** 10^3 leukocytes/jxL of blood is used. The leukocyte count was not available for each blood sample, but the following age-stratified leukocyte counts were used to calculate parasites per microliter of blood: < 4 years, 8.6 **x** 10^3 leukocytes/jxL; 5-9 years, 7.8 **x** 10^3 leukocytes/jxL; > 10 years, 7.7 **x** 10^3 leukocytes/jxL (M. Bruce, unpublished data).

Laboratory methods. *DNA polymorphism analysis.* DNA samples were purified with Glassmilk (Qbiogene Inc., UK) from leukocytes stored in Guanadine-HCl, as recommended by the manufacturer. Hp alleles were determined by polymerase chain reaction (PCR) using 0.025 U/jxL HotStar Taq DNA polymerase (Qiagen) with supplied buffer, oligonucleotide primers (200 nmol/L each), dNTPs (200 (xmol/L each) in two separate reactions as follows: *Hp*^{1}, forward primer (5'-GGGGACAGCTTTTGCAGTGG-3'), reverse primer (5'-GCCGGATTCTTGGGCTTCCC-3') subjected to 94°C for 15 minutes, 35 cycles of 15 seconds at 94°C, 30 seconds at 60°C, and 2 minutes at 72°C; *Hp*^{2}, forward primer (5'-GTTGGAGATAAACTTCCTGAATGTGAAGC-3'), reverse primer (5'-CCACATAGCCATGTGCAATCTCG-3') subjected to 94°C for 15 minutes, 7 cycles of 15 seconds at 94°C, 30 seconds at 60°C, decreasing 0.5°C at each cycle, and 2 minutes at 72°C; 35 cycles of 15 seconds at 94°C, 30 seconds at 57°C, and 2 minutes at 72°C Each individual had either a band corresponding to the *Hp1* allele, a band corresponding to the *Hp2* allele, or two bands if both alleles were present. α^{+}-Thalassemia genotypes were determined in a single-tube multiplex PCR with some modifications.[37] In short, approximately 1 mm^2 of Isocode Stix paper blotted with blood samples was washed with H_2O and directly PCR-amplified with the reaction mixture: 0.05 U/jxL AmpliTaq Gold DNA polymerase (Applied Biosystems) with its supplied buffer, 1.5 mmol/L $MgCl_2$, 200 (xmol/L each dNTP, 1 mmol/L betaine (Sigma), 5% dimethyl-sulfoxide (Sigma), and oligonucleotides a2/3.7-F (400 nmol/L), 3.7/20.5-R (400 nmol/L), a2-R (200 nmol/L), 4.2-F (400 nmol/L), and 4.2-R (400 nmol/L).[37] The reaction was subjected to 94°C for 15 minutes; 35 cycles of 15 seconds at 94°C, 30 seconds at 60°C, and 3 minutes at

72°C. SAO (band 3 deletion) was genotyped by PCR using human DNA from Isocode Stix as described above.[38] G6PD deficiency was evaluated qualitatively (Sigma Diagnostics visual colorimetric assay), and ABO blood groups were determined using anti-A and anti-B antibodies from a kit (BIOSCOT, Edinburgh, UK). The information available for each polymorphism varied because of the lack of enough blood, loss of the sample, or unsuccessful typing.

Hp assay. Plasma Hp levels were determined by enzyme-linked immunosorbent assay (ELISA) as published previously.[39] This method was comparable with results from radial immunodiffusion assays (Dade Baring) using plasma from white controls (R^2 = 0.9022; H. Imrie, unpublished data). The median (range) of Hp levels using the ELISA in these white controls was 1.653 mg/mL (range, 0.05-3.13 mg/mL).

Statistical analysis. Because of the repeated measures study design, appropriate statistical tests were chosen to analyze paired data. The frequency of sex by age in 1999 was assessed by a χ^2 test. Differences in categorical variables between 1999 and 2000 were assessed using McNemar test. Differences in *Plasmodium* density for positive slides between 1999 and 2000 were assessed using a Wilcoxon signed rank test, and those in Hb levels using a paired *t* test. In each case, the analysis only included subjects with data available for both years. Within each year, the frequencies of the categorical malariometric indices by age were assessed by χ^2 tests, that of *Plasmodium* density by a Kruskal-Wallis test, and that of Hb and temperature by analysis of variance. Associations between host polymorphisms and malariometric indices were examined using χ^2 tests, ordinal regression, Kruskal-Wallis tests, and analysis of variance. Finally the associations between *Plasmodium* density, Hb, and genotypes and between Hp levels and the other variables were examined using linear mixed models (equivalent to multiple regressions but adjusting for correlation between the values for the same subject by including a random effect for each subject).

Data considerations. Because the Hp levels showed considerable heteroscedasticity (non-constancy of the variance), the levels were transformed before analysis, using the transformation log(Hp + 0.05). This was chosen by examining profile likelihoods.[40] The *Plasmodium* densities were transformed by *log(Plasmodium* spp. + 1) in the mixed model analyses to reduce the skewness but leaving the zero values unchanged. Additional exclusion criteria for the analysis were those with clinical malaria defined as parasites > 1,000/(xL of blood and temperature > 37.5°C (N = 11 in 1999, N = 23 in 2000).[28]

Details of Hp regression analysis. The linear mixed models were developed using R 1.8.1,[41] and the stepwise search was performed on a data set that omitted all subjects for whom any data were missing. The stepwise procedure, with Akaike's information criterion (AIC) as the criterion of goodness of fit, was commenced at a model including Hp genotype, a^+-thalassemia genotype, presence of splenomegaly, Hb level (continuous variable), and age (continuous variable). The full model examined the effect of *P. falciparum* (four categories or logarithm of density), *P. vivax* (four categories or quadratic effect of logarithm of density), *P. malariae* (two categories or logarithm of the density), and *P. falciparum* gametocytes (two categories or logarithm of density), sex, ABO blood groups, SAO and G6PD genotypes, temperature together with a quadratic term for Hb, and interactions between variables. The final model was refitted to a data set excluding only those subjects with missing data for the variables that remained in the model, and variables with $P > 0.05$ were removed. *P* values reported are adjusted for multiple comparisons using Bonferroni.

SPSS for Windows (version 11.5) was used for the analysis with the exception of the Hp linear mixed model, which was developed in R1.8.1.[41]

The project received ethical approval from the PNG Medical Research Advisory Committee and Institutional Review Board of the New York University School of Medicine.

RESULTS

Full cohort descriptives. *Study subjects.* A serial cross-sectional study took place in the Amele region, Madang, PNG, in November to December 1999 and 2000. After exclusions, the study consisted of 547 individuals (51% boys) 1-17 years of age in 1999. Table 1 shows age-specific and overall frequencies of malariometric indices in both cross-sectional studies in 1999 and 2000. Overall, there was no statistical difference ($P > 0.05$) between malariometric data in the 2 collection years with the exception of *P. vivax* prevalence ($P = 0.04$).

Frequencies of host polymorphisms for the study population are detailed in Table 2. There were no significant associations between any of the host polymorphisms ($P > 0.1$). There was an association between G6PD and sex ($P = 0.009$).

TABLE 1
Age-specific and overall prevalence of malriometric indices

			P. falciparum		*P. vivax*		Splenomegaly	
Survey	Age class (years)	*n*	Prevalence	Density	Prevalence	Density	Prevalence	Hemoglobin (mg/dL)
1999	1-4	94	35.1	3.08 (0.86)	26.6	2.62 (0.63)	23.7	10.07 (1.75)
	5-9	188	39.6	2.78 (0.82)	32.1	2.46 (0.62)	33.5	11.03 (1.78)
	10-17	254	37.4	2.72 (0.71)	26.8	2.19 (0.54)	16.5	11.43 (1.51)
	Total	536	37.8	2.8 (0.78)	28.6	2.36 (0.61)	23.7	11.02 (1.73)
			$P = 0.76$	$P = 0.07$	$P = 0.43$	$P = 0.003$	$P < 0.001$	$P < 0.001$
2000	1-4	70	18.2	2.86 (0.84)	47.3	2.88 (0.79)	30.4	10.01 (1.48)
	5-9	153	33.8	2.87 (0.85)	33.1	2.34 (0.7)	31.4	10.3 (1.43)
	10-17	301	43.2	2.66 (0.74)	12.6	1.95 (0.44)	13.4	11.14 (1.27)
	Total	524	37.2	2.73 (0.78)	23.1	2.36 (0.74)	20.9	10.75 (1.44)
			$P < 0.001$	$P = 0.21$	$P < 0.001$	$P < 0.001$	$P < 0.001$	$P < 0.001$

Continuous variables are shown as mean value (SD). Prevalence is defined as the percentage of subjects with malarial parasites/splenomegaly. Density is $\log_{10}$ value/jxL of positive slides. *P. falciparum* gametocytes were found in 9.5% and 7.8% of samples in 1999 and 2000, respectively.

TABLE 2
Frequency of host polymorphisms

Host polymorphism	Frequency	
	n	(%)
a^+-Thalassemia genotype		
aa/aa	39	(7.1)
-a/aa	201	(36.7)
-a/-a	306	(55.9)
Missing data	1	(0.2)
Haptoglobin genotype		
1-1	201	(36.7)
2-1	240	(43.9)
2-2	104	(19.0)
Missing data	2	(0.4)
ABO blood group		
A	175	(32.0)
AB	55	(10.1)
B	136	(24.9)
O	178	(32.5)
Missing data	3	(0.5)
SAO genotype		
SAO	49	(9.0)
Wild type	497	(90.8)
Missing data	1	(0.2)
G6PD phenotype		
Not normal	55	(10.1)
Normal	469	(85.7)
Missing data	23	(4.2)

Missing data represents unsuccessful genotyping/phenotyping. SAO; Southeast Asian ovalocytosis; G6PD; glucose-6-phospate dehydrogenase deficiency.

Boys had a higher prevalence of G6PD deficiency (14%) compared with girls (7%).

Host polymorphisms and malariometric indices. Host erythrocyte polymorphisms could influence hemolysis and thereby Hp levels. This could occur either directly, where the polymorphism is associated with hemolytic anemia, or indirectly by influencing parasitemia. There were few associations between host polymorphisms and parasite prevalence in the 2 study years. In 1999, both blood group B and Hp genotype were associated with parasite prevalence *(P* = 0.009 and *P* = 0.05, respectively). Blood group B had significantly lower prevalence of *Plasmodium* (54.9%) compared with A (77.8%), AB (77.8%), and O (67.12%), whereas Hp 2-2 had higher prevalences (75.7%) compared with 1-1 (63.8%) and 2-1 (62.4%). In 2000, non-thalassemics had a lower prevalence of *Plasmodium* (37.1%) compared with homozygotes and heterozygotes (61.9% and 62.4%, respectively, *P* = 0.01). There was no association between host polymorphisms and mean parasite density *(P* > 0.05).

Mean Hb levels were significantly associated with a^+-thalassemia after controlling for age and parasite density. a^+-Thalassemia homozygotes had lower mean Hb levels (10.78 g/dL; 95% CI, 10.64, 10.91) compared with heterozygotes (11.04 g/dL; 95% CI, 10.88, 11.21; *P* = 0.04) and non-thalassemics (11.16 g/dL; 95% CI, 10.78, 11.54; *P* = 1.0). Ordinal regression was used to examine associations between host polymorphisms and splenomegaly in both years, 1 year, or neither years. Splenomegaly, was associated with both sex *(P* = 0.026) and SAO *(P* = 0.002) after controlling for age. Girls had a higher prevalence of normal spleens in both studies (71.0%) compared with boys (62.0%). Individuals with SAO had a higher prevalence of splenomegaly in both (19.1%) or one (36.2%) of the studies compared with normals (10.2% and 21.1%, respectively). There was no significant association between host polymorphisms and mean temperature or fever *(P* > 0.05).

Haptoglobin. After exclusions, 547 children contributed 1,060 Hp measurements for the analysis (536 in 1999 and 524 in 2000). Median Hp levels in 1999 were 0.275 (range, 0-3.35) and 0.25 (range, 0-2.85) mg/mL in 2000. There was no significant difference in Hp levels between the 2 years *(P* = 0.34). Ahaptoglobinemia (absence of detectable Hp) was present in 16 individuals in 1999 and 7 individuals in 2000. The prevalence of hypohaptoglobinemia (Hp < 0.18 mg/mL) was found in 39.6% and 38.2% of children in 1999 and 2000, respectively.

Multivariate analysis. A linear mixed model was used to examine the effect of a large number of variables on Hp levels. The resulting fitted mixed model predicts the median Hp level and 95% CIs for the population controlling for relevant variables. The final model chosen included Hp genotype, a^+-thalassemia, splenomegaly, age, Hb (quadratic effect), *P. falciparum*, *P. vivax* (quadratic effect), and gametocyte density. No evidence of interaction between these variables was found.

The overall Hp level in this population after controlling for relevant variables was 0.264 mg/mL (95% CI, 0.216, 0.321). The model predicts a difference in median Hp between the different Hp genotypes (overall *P* = 0.002; Figure 1). The model also showed differences in median Hp according to a^+-thalassemic genotype (overall *P* = 0.011). Homozygotes had significantly higher Hp than heterozygotes (Figure 2). Non-thalassemic individuals had a median Hp level of 0.256 mg/ml (95% CI, 0.186, 0.346), but this did not differ significantly from either homozygotes or heterozygotes *(P* > 0.78). Hp levels decreased with age *(P* < 0.001), decreasing Hb *(P* < 0.001), and increasing parasite densities *(P* < 0.005; Figure 3). Individuals with splenomegaly had median Hp levels of 0.219 mg/mL (95% CI, 0.173, 0.275), which were lower compared with those with normal spleens (0.261 mg/mL; 95% CI, 0.261, 0.382; *P* < 0.001).

DISCUSSION

To determine the influence of malariometric indices and host polymorphisms on Hp concentrations, a serial cross-

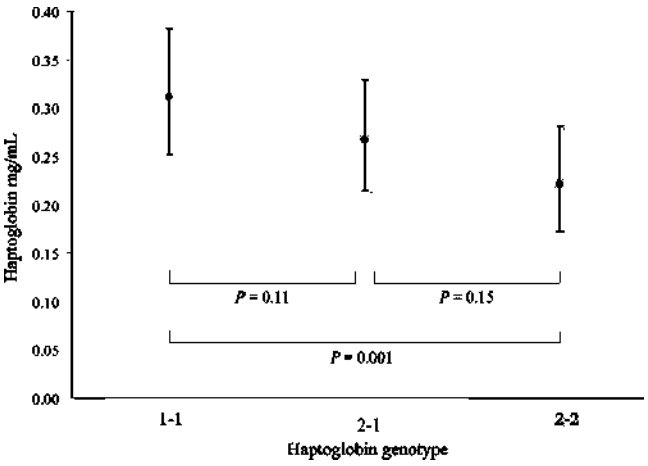

FIGURE 1. Median Hp levels in P N G children according to Hp genotype. Error bars correspond to 95% CIs.

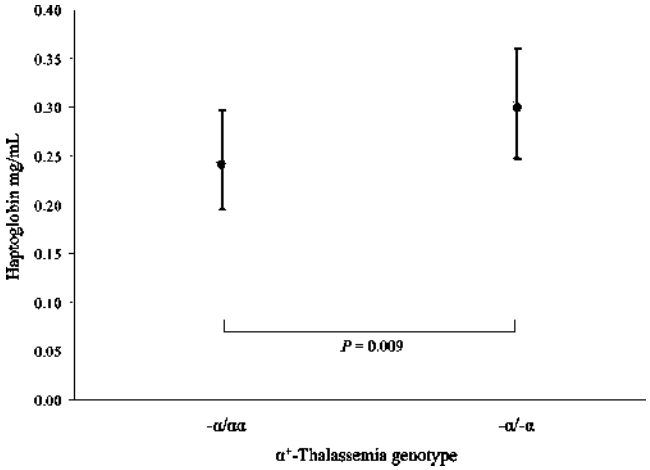

FIGURE 2. Median Hp levels in PNG children according to a^+-thalassemia genotype. Error bars correspond to 95% CIs.

sectional study was undertaken in Madang Province, PNG. Hematological parameters would be expected to vary in an individual exposed to malaria infection. The serial study design ensured that any real difference in Hp levels, with respect to host genetics, would be seen in both years thereby reducing the possibility of these results occurring by chance. Overall, there was no statistical difference between prevalences of malariometric indices between the 2 years, and children showed typical age-related patterns of malaria infection for this area.[25]

The frequency of a^+-thalassemia and other host polymorphisms are very consistent with previous studies in the Amele region.[22,30,32,42,43] Lower prevalences of *Plasmodium* were found in individuals with blood group B and Hp 2-2 in 1999 and non-thalassemics in 2000. These results were not consistent in both years and may therefore have occurred by chance. There was no difference between parasite density and host genotype in this study as assessed by mixed linear models. Furthermore, data from the same children showed that rates of seroconversion to *P. falciparum* variant surface antigens are the same, regardless of host genotype (P.M., unpublished data). Interestingly, splenomegaly prevalence was significantly elevated in ovalocytic subjects. This is not consistent with other studies, but the enlargement of the spleen in these individuals may be explained by the selective removal of ovalocytic erythrocytes by the spleen.[32,43] a^+-Thalassemia homozygotes had lower mean Hb levels compared with heterozygotes. This is a common consequence of this genotype, which experiences reduced erythrocyte indices and, in particular, hemolytic anemia.[20,21] G6PD deficiency was more prevalent in boys in this study. The *G6PD* gene is on the X chromosome, and sex differences have been previously noted.[44]

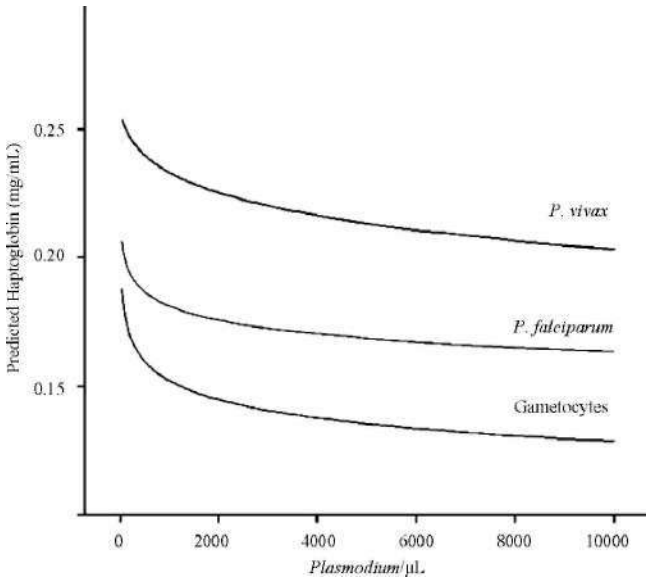

FIGURE 3. Predicted Hp levels according to *Plasmodium* spp. density. Hp levels decrease with increasing parasite density ($P < 0.005$).

Multivariate analysis showed that levels of Hp were related to Hp genotype, age, total Hb, parasite density, splenomegaly, and a^+-thalassemia genotype. The relationship between Hp levels and Hp phenotype has been noted by others.[6] Hp2-1 and 2-2 are polymeric, whereas Hp1-1 is a tetramer.[3] The binding of one Hb molecule to one molecule of a polymeric form of Hp and its subsequent clearance would result in a greater relative reduction in the total pool of Hp available compared with the clearance of one Hp1-1 tetramer. Furthermore, clearance of 2-2 complexes of Hb-Hp exhibit higher functional affinity for CD163 than do complexes of Hp1-1-Hb.[2]

Hp levels were found to decrease with age from 1 to 17 years in this cohort. A study in a malaria endemic area of The Gambia also found levels decrease with age to 10 years.[23] Hp concentration was positively correlated with Hb levels and inversely related to parasite density of all *Plasmodium* spp., presumably because of the rapid clearance of the Hp-Hb complex during hemolysis. Levels were highest in children infected with *P. vivax,* because *P. vivax* densities were lower in these children. High parasite densities and possibly mass hemolysis of infected erythrocytes may induce gametocytogenesis.[4] This may explain why Hp levels were lowest in children with *P. falciparum* gametocytes. Hp levels were also reduced in the presence of splenomegaly, which occurs in children experiencing high-density malaria infection in the Amele population.[2,28]

Our data also showed that levels of Hp varied with a^+-thalassemia genotype. There was no significant difference between non-thalassemics and either heterozygotes or homozygotes. This was probably because of the low frequency of non-thalassemics in the study population resulting in a high variance in the estimates of their median Hp levels. Median levels in non-thalassemics (0.264 mg/mL; 95% CI, 0.216, 0.321) were lower than that found in whites (1.653 mg/mL; range, 0.05-3.13 mg/mL; H. Imrie, unpublished observations) of the same age; this is because of hemolysis experienced during chronic malaria infections that children in this area harbor.[29,46] The levels in this study fall within the range of that previously reported in other areas of PNG.[47,48] After stratification for Hp phenotype, one study showed levels comparable with ours in the lowlands of PNG but higher levels in the highlands where malaria is absent or epidemic.[47]

Hp levels were significantly higher in individuals who were homozygous compared with heterozygous for a^+-thalassemia. This finding was unexpected; in a^+-thalassemia homozygos-

ity, the primary pathophysiology is that of increased peripheral hemolysis marked in this study by the decreased Hb in those with this genotype.[18,20] Thus, reduced levels of Hp would be predicted in homozygotes compared with heterozygotes because of increased clearance of Hb. This result could be explained by differences in 1) parasitemia, 2) mean cell hemoglobin (MCH), or 3) increased production of Hp by homozygotes compared with heterozygotes.

A possible explanation for the higher levels of Hp in a^+-thalassemic homozygotes is that they experience fewer *Plasmodium* infections than heterozygotes. This would reduce hemolysis and increase Hp levels. There were no significant differences in either *Plasmodium* prevalence or density between the a^+-thalassemia genotypes. This is consistent with other studies in the area.[22]

Alternatively, hematological parameters could explain the result. Homozygotes have lower MCH.[18] Therefore, after Hp-Hb clearance, more Hp would be present in the plasma per erythrocyte lysed. However, the relative contributions of MCH and hemolysis, associated with individuals with a^+-thalassemia homozygosity, and Hp levels is unknown, particularly in the presence of malaria.

The hematological abnormality associated with a^+-thalassemia homozygosity could also cause elevated production of Hp. The hemolytic anemia associated with a^+-thalassemia may cause an elevated production of Hp by hepatocytes through interleukin (IL)-6 because of the effects of free Hb.[49,50] Also, Hb is rapidly transformed into methemoglobin, within the circulation, which acts directly on endothelial cells causing them to secrete IL-6.[51] IL-6 stimulates the release of Hp by hepatocytes. In addition, while acute, severe hemolysis (e.g., that associated with clinical malaria) will always result in a reduction of Hp because of clearance; the outcome of chronic, low-level hemolysis (e.g., that associated with a^+-thalassemia and/or subclinical malaria) is harder to predict, being a balance between the loss of Hp through clearance and increased production caused by IL-6.

In conclusion, we showed that a^+-thalassemia homozygotes have increased levels of Hp during asymptomatic malaria infection. This may be because of differences in MCH- or IL-6-dependent responses. The potential role of acute phase proteins in the role of a^+-thalassemia-mediated protection has yet to be elucidated. Whether acute phase proteins act as a possible marker, contributor to, or effector for this protection warrants further investigation. Additional studies are also required to explore differences in Hp levels in thalassemics outside of PNG and malaria-endemic areas.

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