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Hemoglobin C is associated with reduced *Plasmodium falciparum* parasitemia and low risk of mild malaria attack

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Genetic predisposition to malaria has been shown by epidemiological, case–control and linkage studies. In particular, case–control studies have recently shown association between hemoglobin C and resistance to severe malaria in Mali and to clinical malaria in Burkina Faso. In a longitudinal study of families living in an endemic area, we investigated whether hemoglobin C is associated with reduced *Plasmodium falciparum* parasitemia and low risk of mild malaria attack. We surveyed 256 individuals (71 parents and 185 sibs) from 53 families during 2 years. Hemoglobin C carriers had less frequent malaria attacks than AA individuals within the same age group ($P = 0.01$). Since age correlated with malaria attack and parasitemia ($P < 0.0001$), we took age into account in association analyses. We performed combined linkage and association analyses, which avoid biases due to population structure. Using multi-allelic tests, we evidenced association between hemoglobin genotype and phenotypes related to malarial infection and disease ($P < 0.001$). We further analyzed individual hemoglobin alleles and detected negative association between hemoglobin C and malaria attack ($P = 0.00013$). Analyses that took into account confounding factors confirmed the negative association of hemoglobin C with malaria attack ($P = 0.0074$) and evidenced a negative correlation between hemoglobin C and parasitemia ($P = 0.0009$). These associations indicate that hemoglobin C reduces parasitemia and confers protection against mild malaria attack.

INTRODUCTION

The complex genetic control in human malaria reflects thousands of years of selective pressure. Hemoglobinopathies were the first polymorphisms thought to be selected under the pressure of malaria. On the basis of the worldwide distribution of hemoglobin S (HbS) and α-thalassemia, Haldane (1) and Allison (2) suggested that α-thalassemia and HbS gave a selective advantage for survival in malaria-endemic areas. Case–control studies have shown the association of resistance to severe malaria with both α-thalassemia (3) and HbS (4,5). Case–control studies have been performed to investigate the role of other candidate genes in severe malaria. Associations have been found between resistance and genes encoding other red blood cell proteins (Band 3, G6PD) or immunological molecules (HLA-B, HLA-DR, TNFα, ICAM1, CD36, iNOS and IFNR) (6,7). It should be stressed that some associations have not been confirmed, suggesting that genetic control in human malaria may differ between populations. Another explanation would be that population associations may occur in the absence of linkage as a result of admixture, heterogeneity or stratification in a population. In this case, association between genes and resistance to malaria does not mean that the candidate genes associated are involved in human malaria. To circumvent the problem of population structure, linkage and association analyses should be performed (8). Such methods have been successfully used to analyze genetic control of parasitemia (9,10) and of mild malaria (11,12).

Strikingly, most of the genes associated with resistance to severe malaria are not associated with mild malaria or parasitemia (6,7). Malaria pathogenesis is incompletely known and the identification of genes controlling phenotypic variations related to malaria infection should be helpful in understanding the mechanisms involved. In particular, this may clarify the relationship between parasitemia and clinical malaria, and between mild malaria and severe malaria.

Several case–control studies have tested the association between hemoglobin C (HbC) and resistance to clinical

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malaria. Results from a study in Nigeria and one in Mali indicated lack of protection (13,14), while association between heterozygosity for C and protection against severe malaria was detected in another study in Mali (15). Recently, a large case–control study in Burkina Faso detected a strong association between resistance to clinical malaria and HbC in both the heterozygous and the homozygous state (16). However, case–control or cross-sectional studies did not detect association of HbC with parasitemia (15–17).

The aim of the present study was to test association in the presence of linkage between hemoglobin genotype on the one hand and parasitemia and risk of mild malaria attack on the other hand. A longitudinal study was conducted over 2 years on 256 subjects from 53 families living in an urban area in Burkina Faso. We report here the results of family-based association analyses.

RESULTS

Hemoglobin genotypes and phenotypes were available for 256 individuals (71 parents and 185 sibs) belonging to 53 families with between two and nine members. The frequencies of hemoglobin A, C and S were respectively 0.814, 0.139 and 0.047 for all individuals. AA, AS, AC and CC genotype frequencies were in Hardy–Weinberg equilibrium ($P = 0.45$). For sibs, Table 1 shows the age distribution among the different hemoglobin groups. The age distributions of AA sibs and sibs with HbC were similar ($\chi^2 = 2.1$, df = 4, $P = 0.71$). Table 2 shows the frequencies of genotype AA, AS, AC and CC in affected sibs. Forty-seven percent of AA sibs, 22% of AS sibs and 25% of AC sibs presented at least one malaria attack (P1) during the study: hemoglobin genotypes, therefore, appeared to influence the occurrence of malaria attack ($\chi^2 = 9.1$, df = 2, $P = 0.01$). Figure 1 shows that HbC carriers had less frequent malaria attacks than AA individuals belonging to the same age class. Since the number of children in the youngest age class was low (Table 1), children aged 1–5 years were excluded in some analyses. The logistic regression, which included the 6–10, 11–15, 16–20 and 21–25 age classes as explanatory variable, showed that the association between HbC and the occurrence of malaria attack was significant ($\chi^2 = 6.1$, df = 1, $P = 0.013$). When children aged 1–5 years were included in the analysis, the negative association of HbC with malaria attack was also found ($\chi^2 = 6.2$, df = 1, $P = 0.012$). The logistic regression treating age as a continuous variable led to very similar results ($\chi^2 = 5.8$, df = 1, $P = 0.016$). The odds of malaria attack between AA individuals and HbC carriers was 2.7 (95% confidence interval 1.2–6.1).

Age correlated with the four phenotypes: malaria attack (P1), risk of developing malaria attack (P2), mean of adjusted parasitemia (P3) and maximum parasitemia (P4) (Table 3). Fifty-four percent (7/13) of sibs 0–5 years of age, 60% (28/47) of those 6–10 years of age, 40% (22/55) of those 11–15 years of age, 35% (14/40) of those 16–20 years of age and 7% (2/30) of those 21–25 years of age had at least one malaria attack (P1) during the study. As expected, age influenced the occurrence of malaria attack ($\chi^2 = 22.9$, df = 4, $P = 0.00013$). Logistic regression considering age as explicative variable on the probability of malaria attack (P2) also showed a highly significant effect of age in the sample of 185 sibs (Table 4). Age also had an effect on mean adjusted parasitemia (P3) and maximum parasitemia (P4) in the sample of 185 sibs (Table 4). Polynomial regression analysis showed that the best fitting function was linear. The regression lines accounted for 14 and 21% of the variance of P3 and P4, respectively. Hence, age was retained for combined linkage and association analyses.

Table 5 shows the results of multi-allelic tests for binary and quantitative phenotypes. Hemoglobin was associated with P1, P2, P3 and P4. The FBAT results showed a deficit transmission of HbC from parents to the offspring with malaria attack. HbC was negatively associated with malaria attack ($Z = -3.82$; $P = 0.00013$). In the same analysis, HbA was positively associated with malaria attack ($Z = 4.27$; $P = 0.00002$). Similar analyses with HbS did not evidence a significant association with malaria attack. The trend, however, was negative, and we lumped together HbS and HbC in some association analyses. We evidenced negative association of grouped alleles S and C with malaria attack ($Z = -3.94$; $P = 0.000082$).

We also tested the association between individual hemoglobin alleles and quantitative phenotypes. Table 6 shows the QTDT results for phenotypes, for which we evidenced association using multi-allelic tests. HbC was found to be negatively associated with P2 ($P = 0.0074$), P3 ($P = 0.0012$) and P4 ($P = 0.0009$) (Table 6). We also found a trend in favor of a protective effect of HbS, although it was not significant. Since our data suggested that both HbS and HbC protect against malaria, we grouped alleles S and C in further analyses. We further found evidence of associations with P2 ($P = 0.0007$), P3 ($P = 0.0001$) and P4 ($P = 0.0003$).

DISCUSSION

In this study, we used family-based association tests to investigate the association between HbC on the one hand and parasitemia and malaria attack on the other hand. Several phenotypes related to malaria infection and disease were tested for association with HbC.

Thirty-five percent of the sibs were carriers of either HbS or HbC. The gene frequency of HbC was higher than the frequency of HbS. This is consistent with the high frequency of HbC in West Africa (15,17,18). The frequencies of AA, AS and AC individuals reported in central Burkina Faso by Modiano *et al*. (16) are very close to the frequencies we report here in a population in the West of Burkina Faso.

Raw data indicated that AS and AC individuals less frequently develop mild malaria attack than AA individuals. The protective effect of HbS on severe malaria (4) and on mild malaria (19,20) was previously reported. Association of HbC with severe malaria has been found in Mali (15) and association of HbC with clinical malaria (severe and mild malaria) has been found in Burkina Faso (16). In the latter study, no difference in HbC was observed between severe and mild malaria, suggesting that HbC also protects against mild malaria. To address this issue, we performed family-based association analyses. We evidenced a strong association between HbC and protection against mild malaria (P1). In our study area, the occurrence of malaria attack slightly decreased with increasing age in individuals less than 20 years of age, as previously described in areas with similar

Table 1. Age distribution of Hb genotype

Age	1 to 5	6 to 10	11 to 15	16 to 20	21 to 25	All
AA	7 (6%)	30 (25%)	33 (27%)	30 (25%)	21 (16%)	121 (100%)
AC and CC	5 (11%)	11 (24%)	13 (28%)	8 (17%)	9 (20%)	46 (100%)
AS	1 (6%)	6 (33%)	9 (50%)	2 (11%)	0 (0%)	18 (100%)
All	13 (7%)	47 (25%)	55 (29%)	40 (22%)	30 (17%)	185 (100%)

Age distribution is given for sibs. CC individuals were 9 and 24 years old.

Table 2. Clinical data of sibs

Genotype	Number of unaffected sibs	Number of affected sibs[a]	All sibs
AA	64 (57.1%)	57 (78.1%)	121 (65.4%)
AS	14 (12.5%)	4 (5.5%)	18 (9.7%)
AC	33 (29.5%)	11 (15.1%)	44 (23.8%)
CC	1 (0.9%)	1 (1.3%)	2 (1.1%)
All	112 (100%)	73 (100%)	185 (100%)

[a]Sibs with at least one malaria attack during the 24 months of the study.

malaria transmission intensities (21). The influence of age was, nevertheless, highly significant. We, therefore, took age into account in further analyses and we confirmed the association between HbC and protection against mild malaria (P2).

Since high parasitemia strongly enhances the risk of malaria attack, we further analyzed the association between Hb genotypes and mean of adjusted parasitemia (P3). We found a negative association between HbC and P3. Our findings contrasted with case–control and cross-sectional studies, which showed no correlation of HbC heterozygosity with parasite rates and densities (15–17). It should be stressed, that we report here a longitudinal and familial study. Because of the fluctuation of parasitemia, longitudinal studies are likely required to address this issue.

Studies that evaluated association between HbS heterozygosity and parasite density also showed contrasting results: parasite density in AS individuals was lower (2,17,19), similar (22) or higher (23) when compared with parasite density in AA individuals. It was proposed that HbS may limit the expansion of malarial infection at high parasitemia, since AS individuals with high parasitemia were rarely found (5,17,18). A similar effect of HbC was also suggested (17,18). In this case, the search for maximum parasitemia in a longitudinal survey may be useful.

We therefore designed another phenotype that may better reflect potential bursts of parasite multiplication: maximum parasitemia (P4). P4 was a quantitative phenotype and represented the highest parasitemia in each individual. Raw data suggested that AA individuals may develop higher maximum parasitemia than did AS, AC and CC individuals. We took into account the influence of confounding factors, such as age, in further analyses. Results of multi-allelic QTDT showed a clear association between hemoglobin genotypes and maximum parasitemia (P4). HbC was negatively associated with maximum parasitemia.

These results clearly indicate that parasite expansion is inhibited in individuals with HbC. This is consistent with *in vitro* studies showing lower parasite multiplication rates in CC than in AA red cells (24–26). These abnormal cells probably represent a barrier for the parasite because of their inability to lyse and release merozoites at the appropriate stage (25). In addition, ring forms and trophozoites showed evidence of disintegration within a subset of CC red cells (26). Although AC red cells sustain normally the growth of *P. falciparum in vitro* (24,25), our findings clearly show a protective effect of heterozygosity for HbC against the parasite. It seems very likely that the *in vivo* protective effect of HbC depends on factors, which are not in the *in vitro* culture system. In particular, the protective effect of HbC may act in synergy with specific acquired immunity, as suggested for the protective effect of HbS (19,27,28).

Whatever the mechanisms involved, we propose that the inhibitory effect of HbC on parasitemia may partly explain the protective effect of HbC on mild malaria. Carriers of HbC, who have a reduced parasitemia, would present a diminished risk of mild malaria attack. One might also assume that the protective effect of HbC against the parasite may participate in the protective effect of HbC against severe malaria.

In our study, HbS was not significantly associated with reduced parasitemia and low risk of malaria attack. Nevertheless, the frequency of HbS was too low to detect a significant association and our results are not in contradiction to previous reports (19,20). Furthermore, we found a negative trend in favor of a protective effect of HbS against malaria, and we evidenced association of grouped alleles S and C with reduced parasitemia and low risk of malaria attack.

In conclusion, the main results of the family-based association analyses showed that HbC is associated with low parasitemia and protection against mild malaria. Our results are in line with those of others who have well established the protection afforded by HbS. Strikingly, the HbC and the HbS mutations occur in the 6th position in the β-globin DNA sequence, and both mutations coexist in several populations in West Africa. Since HbC appears to have few adverse effects, compared with HbS in the homozygous state, it has been suggested that HbC would replace HbS in West Africa (17).

MATERIALS AND METHODS

Subjects

The study subjects live in an urban district of Bobo-Dioulasso, the second largest town of Burkina Faso, in an endemic area for malaria. *P. falciparum* transmission occurs only during the rainy season (August–December). During the 2 years of the study, the entomological inoculation rate was close to

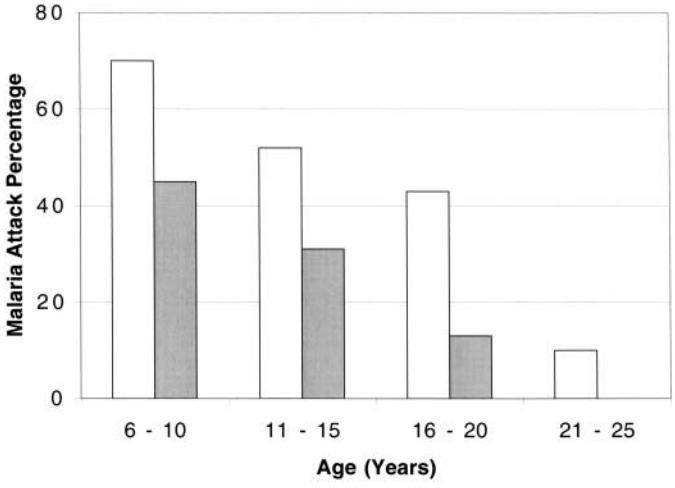

Figure 1. Percentage of malaria attack according to age in AA group (□) ($n = 114$) and in AC and CC group (■) ($n = 41$). AA ($n = 7$) and AC sibs ($n = 5$) aged less than 5 years were not included. The inclusion of the sibs aged 1–5 years in the analysis did not alter the distribution. Within the 1–10 age class, 67.6% of AA individuals and 43.7% of HbC carriers were affected.

30 infective bites per year. The study population and the area of parasite exposure have been described elsewhere (29). The study included 256 individuals (71 parents and 185 sibs) from 53 families; the mean age of the sibs was 13.0 ± 5.4 (range 1–24). Most of the population belongs to the Mossi ethnic group (50%); the other groups included the Dafing (19%), Guian (5%), Bissa (15%), Samogo(1%), Bobo (6%) and Nounouma (4%). The seasonal intensity of parasite transmission was homogeneous within the area. The whole population volunteered to participate in the study, and all participants were clearly informed of the objective and the protocol. The Expert Committee of the University of Ouagadougou and the Medical Authority of Burkina Faso approved the study protocol.

Parasitological examination

Determination of parasitemia was described in our previous study (30). Briefly, each family was visited 20 times during the 24 months of the study and parasitemia was measured. In addition, parasitemia was measured during febrile episodes. The mean number of parasitemia measurements per subject was 13.4 ± 5.5 (range 1–25). Fingerprint peripheral blood samples were taken from all family members present and thick and thin blood films were stained with Giemsa. The parasite determination and numeration were established blindly from two independent readings. Only *P. falciparum* asexual forms were retained to determine parasitemia. Parasitemia was defined as the number of parasitized erythrocytes observed per µl in thin blood films.

Clinical data

Febrile episodes were extensively recorded by active case detection over 24 months. For patients with fever, a thick blood film was prepared by the standard procedures. Diagnosis of mild malaria attack was based on *P. falciparum* parasitemia, fever (axillary temperature more than 37.5°C) and clinical symptoms (headache, aching, vomiting or diarrhea in children); in that case no threshold of parasitemia was used. In the absence of classical symptoms of malaria, and once other pathologies could not be eliminated, only children (age <15 years) with more than 5000 parasites per µl and older subjects with more than 2000 parasites per µl were considered as having had a malaria attack. According to the recommendation of the CNRFP (Centre National de Recherche et Formation sur le Paludisme) of Burkina Faso, each episode of illness was treated with 25 mg/kg chloroquine over 3 days or until recovery. Parasitemia was checked at the end of the treatment. Eighty individuals (seven parents and 73 sibs) presented at least one mild malaria attack during the survey. No cases of severe malaria were recorded.

Determination of phenotypes

Table 3 describes the phenotypes used in the study.

Mild malaria attack (P1) was directly based on clinical data. Subjects who presented at least one mild malaria attack during the survey were considered affected. The others were considered unaffected.

The second phenotype (P2) was based on the risk of developing malaria attacks. To take into account the influence of covariates on mild malaria attack, we performed logistic regression. Age was considered a continuous variable. The logit of the probability P of malaria attack can be expressed in the form of function of age as follows: $\log(P/1-P) = \beta 0 + \beta a\text{Age}$, where $\beta 0$ is constant and βa is the regression coefficient (31). The residual of the logistic regression model, which considered age a continuous variable (P2) was used in association and linkage analyses. In some analyses, age was also categorized into five classes.

Mean of adjusted parasitemia (P3) was a logarithmic transformation of the parasitemia adjusted for seasonal transmission (30). All the parasitemia data were included. To take into account the seasonality of the transmission, the influence of the date of the visits on $\ln(1 + \text{parasitemia})$ (LP) was evaluated by one way analysis of variance. The mean LP observed during each visit was calculated. The individual LP was then corrected for the visit effect by subtracting from each individual LP the mean LP of the corresponding visit. Multiple polynomial regression was done with age and the number of measurements. The explicative variables were treated as continuous variables. The number of measurements did not correlate with P3. In contrast, age strongly correlated with P3 and was retained as a covariate for linkage and association analyses.

Maximum parasitemia (P4) was based on a logarithmic transformation of the highest parasitemia in each individual. Multiple polynomial regression was done as indicated for P3. The analysis revealed that age and the number of measurements had an effect on P4 ($P = 0.0001$). We took into account age and the number of measurements in association and linkage analyses.

All computations were done with SPSS software (SPSS, Boulogne, France). Tests of the hypothesis that a regression coefficient was 0 were done with the Wald χ^2 statistic for logistic regression and Student's *t*-test for multiple polynomial regression. Only terms significant at the 5% level were retained.

Table 3. Phenotypes related to malarial infection and disease

Phenotype	Type of phenotype	Covariates	Distribution of the residual
Malaria attack (P1)	Binary	—	—
Risk of developing malaria attacks (P2)	Quantitative	Age	Non-normal
Mean of adjusted parasitemia (P3)	Quantitative	Age	Normal
Maximum parasitemia (P4)	Quantitative	Age, number of measurements	Normal

Table 4. Correlation[a] of P2, P3, P4 with age[b]

Phenotype	Slope	SD	*P*
P2[c]	−0.123	0.028	1.3×10^{-5}
P3[d]	−0.008	0.001	8.5×10^{-8}
P4[d]	−0.045	0.006	2.5×10^{-11}

[a]The analysis included only sibs.
[b]Age was treated as a continuous variable in each model.
[c]Logistic regression was used; the *P* value was calculated by using the Wald χ^2 statistic.
[d]Linear regression was used; the *P* value was calculated by using Student's *t*-test.

Table 5. Multi-allelic[a] tests[b] of association for hemoglobin polymorphisms

Phenotype	Covariates	χ^2 (df)	*P*
P1	—	18.34 (2)	0.0001
P2	Age	—	0.0001[c]
P3	Age	14.61 (2)	0.0007
P4	Age	17.02 (2)	0.0002
	Age, number of measurements	13.80 (2)	0.001

[a]Alleles A, S and C were simultaneously analyzed.
[b]FBAT and QTDT were used for binary phenotype and for quantitative phenotypes, respectively.
[c]Empirical *P* value for non-normal data.

Table 6. Association of alleles with quantitative phenotypes

Phenotype (covariates)[a]	Allele	χ^2 (df)	*P*	Direction of association
P2 (Age)	A	—	0.0007[b]	Positive
	C	—	0.0074[b]	Negative
P3 (Age)	A	15.18 (1)	0.0001	Positive
	C	10.54 (1)	0.0012	Negative
P4 (Age, number of measurements)	A	13.41 (1)	0.0003	Positive
	C	11.05 (1)	0.0009	Negative

[a]Covariates that influence the phenotypes are taken into account using QTDT.
[b]Empirical *P* values for non-normal data.

Hemoglobin typing

Blood samples were taken from 256 individuals by venipuncture. The hemoglobin genotypes were identified by electrophoresis of red blood cell lysates on acetate membrane at an alkaline pH. Acetate sheets were stained with ponceau red, yielding discrimination of hemoglobins A, S and C.

Combined linkage and association analysis

Association in the presence of linkage was assessed using family-based tests, which avoid biases due to population stratification, population heterogeneity, or population admixture, and are designed to deal with multiple alleles.

Association and linkage of binary trait were assessed using the FBAT program (32). The FBAT statistics, which uses data from sibships in nuclear families takes into account sibling correlations. The default null hypothesis tested is no linkage and no association. The statistics under this hypothesis calculated under the distribution of offspring genotype are conditional on parental genotype and on trait values for offspring and parents. FBAT calculates a *Z* score and a 2-side *P* value based on a normal approximation.

Combined association and linkage analyses of quantitative traits were carried out using the orthogonal model released in the QTDT 2.3.0 program (33). Variance components are used to construct a test that utilizes information from all available offspring. It is a general linkage-disequilibrium test that is applicable to the analysis of quantitative traits in nuclear families of any size, with or without parental genotypes. Evidence of association can be evaluated by likelihood-ratio test (null hypothesis likelihood L0 versus alternative hypothesis likelihood L1). Asymptotically, the quantity 2(lnL1-lnL0) is distributed as χ^2 with df equal to the difference in number of parameters estimated. Because variance components can be sensitive to the phenotypic distribution, we checked the hypothesis of multivariate normality with the SPSS software (SPSS, Boulogne, France). Violation of multivariate normality warrants the Monte-Carlo permutation test. In this case, we performed 9999 permutations to calculate empirical *P* values.

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