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ASSOCIATION OF FCγ RECEPTOR IIA (CD32) POLYMORPHISM WITH MALARIAL ANEMIA AND HIGH-DENSITY PARASITEMIA IN INFANTS AND YOUNG CHILDREN

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Abstract. Protective immunity against *Plasmodium falciparum* is partially mediated through binding of malaria-specific IgG antibodies to Fcγ receptors. Polymorphic variability in FcγRIIa (H/R-131) is associated with differential binding of IgG subtypes and malaria disease outcomes. However, the role of FcγRIIa-131 variability in conditioning susceptibility to severe malarial anemia, the primary manifestation of severe malaria in holoendemic *P. falciparum* transmission areas, is largely undefined. Thus, FcγRIIa-H131R polymorphism was investigated in 493 children who came to a hospital with acute malaria. Variation in FcγRIIa-131 was not significantly associated with severe malarial anemia (hemoglobin [Hb] < 6.0 g/dL) or malaria anemia (Hb < 8.0 g/dL). However, relative to the heterozygous genotype, homozygotes for the R131 alleles were protected against high-density parasitemia (≥ 10,000 parasites/μL; odds ratio [OR] = 0.58, 95% confidence interval [CI] = 0.37–0.92, $P = 0.02$), while homozygotes for the H131 alleles were mildly protective (OR = 0.71, 95% CI = 0.45–1.13, $P = 0.14$). Additional multivariate analyses showed that infection with human immunodeficiency virus type 1 did not influence the associations between FcγRIIa-H131R polymorphism and malaria disease outcomes. Genotypic results presented here parallel data illustrating that parasite density is unrelated to the severity of anemia in children with acute malaria. Thus, although homozygosity for the R131 allele protects against high-density parasitemia, FcγRIIa-131 polymorphism does not protect against malaria anemia.

INTRODUCTION

One of the most common causes of morbidity and mortality in African children is *Plasmodium falciparum* malaria in which severe manifestations of disease are defined by one or more of the following: high-density parasitemia (HDP), hypoglycemia, cerebral malaria (CM), severe malarial anemia (SMA), and respiratory distress. Among these disease sequelae, SMA is responsible for the greatest degree of malaria-associated morbidity and mortality worldwide.[1]

Naturally acquired antibodies are important for protection against asexual blood stages of malaria as shown by passive transfer of immunoglobulin (IgG) from malaria-immune adults to malaria-naive children.[2] Binding of the heavy chain immunoglobulin domain to Fc receptors on phagocytic cells is important for protective immunity against malaria. FcγRIIa (CD32) is a predominate low-affinity Fcγ-receptor expressed on monocytes and macrophages, and other immune cells, that binds to all IgG subtypes (IgG_{1-4}),[3] thereby providing an important link between humoral and cellular malarial immunity.

A non-synonymous single nucleotide polymorphism in FcγRIIa that changes a histidine (H) to an arginine (R) residue at position 131 is associated with differing susceptibilities to severe malaria. Previous studies in a holoendemic *P. falciparum* transmission area of western Kenya showed that the R/R131 genotype that preferentially binds IgG_1 and IgG_3[4] is protective against parasitemia (> 5,000 parasite/μL) relative to the H/R131 genotype.[5] Additional investigations in The Gambia demonstrated that homozygous H131 alleles that preferentially bind IgG_2[6] increase susceptibility to malaria in children with a mixed clinical phenotype of severe disease: CM, SMA, and/or hypoglycemia.[7] In contrast, although none of the FcγRIIa-131 genotypes were associated with susceptibility to CM in Thai adults, haplotype analysis showed that the FcγRIIIB-NA2 and FcγRIIa-HH131 alleles together increased susceptibility to CM.[8] Defining the association between FcγRIIa-131 genotypes and malarial anemia (MA) in infants and young children residing in holoendemic *P. falciparum* transmission areas is important because this clinical phenotype accounts for the largest degree of malaria-induced morbidity and mortality. The role of FcγRIIa-131 polymorphism in regulating susceptibility to SMA (hemoglobin [Hb] < 6.0 g/dL) and MA (Hb < 8.0 g/dL) was therefore examined in children less than three years of age who came to a hospital in a rural holoendemic *P. falciparum* area of western Kenya. The association between FcγRIIa-131 polymorphism and HDP (≥ 10,000 parasites/μL) was also determined.

METHODS

Study participants. A total of 493 children were recruited as part of an ongoing study of SMA at Siaya District Hospital in western Kenya. Data presented are for the cross-sectional analyses in which study participants had their first hospital contact for malaria. In this holoendemic area of *P. falciparum* transmission,[9] SMA and hyperparasitemia are the most common clinical manifestations of severe malaria, with CM occurring only in rare cases.[10] Greater than 99% of the study participants were from the Luo ethnic group. Definitions of disease severity were based on a previous large-scale longitudinal study examining the distribution of Hb concentrations in children less than four years of age in western Kenya.[11] Children were categorized into four groups based on the pres-

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ence of parasitemia (any density) and severity of anemia (Table 1). Administration of antimalarials and appropriate supportive therapy was provided to all children as per Kenya Ministry of Health guidelines.

In addition, human immunodeficiency virus type 1 (HIV-1) status was determined in all study participants by two rapid serologic antibody tests (Unigold™; Trinity Biotech Lc., Bray, Ireland and Determine™; Abbott Laboratories, Inc., Abbott Park, IL) and HIV-1 DNA polymerase chain reaction (PCR) analysis. The prevalence of HIV-1 based on PCR-positive results, confirmed on two separate blood samples, was 4.5%. Trimethoprim-sulfamethoxazole was administered to all children who had positive results in one or both serologic tests. None of the HIV-1-positive study participants had been started on antiretroviral treatment at the time of sample collection. Written informed consent in the language of choice (i.e., English, Kiswahili, or Dholuo) was obtained from the parents or guardians of participating children. Pre- and post-test HIV counseling was provided for all participants. The study was reviewed and approved by the ethics committees of the University of Pittsburgh Institutional Review Board and the Kenya Medical Research Institute Ethical Review Board.

Laboratory procedures. Parasitemia and Hb concentrations were determined in heel or finger prick blood (< 100 μL) and venipuncture samples (< 2.0 mL), respectively. Asexual malaria parasites were counted against 300 leukocytes in peripheral blood smears stained with Giemsa, assuming a count of 8,000 white blood cells/μL of blood. Hemoglobin concentrations were determined on a Beckman Coulter AcT diff2™ (Beckman-Coulter Corporation, Miami, FL).

Diagnosis of HIV-1 status. Exposure to HIV-1 was determined through the use of two rapid serologic test methods (Unigold™ and Determine™). Children with single- or double-positive serologic results were further evaluated for HIV-1 infection by a nested PCR of proviral DNA extracted from dried blood spots collected on foam tipped applicators (FTA) classic cards (Whatman Inc., Florham Park, NJ). HIV-1 *gp41* primers were selected for highly conserved HIV-1 group M, N, and O sequences for use in western Kenya.[12,13] The PCR results were confirmed on fresh samples approximately three months after the initial test.

Genotyping. DNA was extracted from blood spots using the Chelex method.[14] FcγRIIa-131 genotypes were determined using previously described methods by gene-specific PCR amplification on a PTC-100™ thermocycler (MJ Research Inc.), followed by allele-specific restriction enzyme digestion with *Bst* UI (New England Biolabs, Beverly, MA).[15] The *Bst*UI digestion of the PCR product (366 basepairs) produced a 322-basepair fragment for the RR131 genotype, a 343-basepair fragment for the HH131 genotype, and both fragments for the HR131 genotype.

Statistical analyses. Statistical analyses were conducted using Minitab Release 13.32 (Minitab Inc., State College, PA). Kruskal-Wallis tests were used for multiple group comparisons and differences between proportions were determined by chi-square analyses. Logistic regression controlling for age, sex, and prevalence of sickle-cell trait (HbAS), was used to examine the association of FcγRIIa-131 polymorphism with SMA, MA, and HDP. Additional multivariate analyses controlling for HIV-1 infection were also conducted to determine the impact of HIV-1 on the association between FcγRIIa-131 polymorphism and malaria disease outcomes. Based on a previous study that reported more than 10,000 longitudinal Hb measurements in an age- and geographically matched reference population,[11] SMA was classified as an Hb concentration < 6.0 g/dL and any density parasitemia. Malarial anemia was defined as an Hb concentration < 8.0 g/dL and any density parasitemia because this criteria identifies those children at greatest risk of malaria-related morbidity and mortality,[16] while HDP was based on a standard definition with parasites/μL ≥ 10,000. The HR131 genotype was used as the reference in the logistic regression analyses because it was the most prevalent genotype in the population. Statistical significance was defined as $P \leq 0.05$.

RESULTS

Characteristics of study participants. A total of 493 children with acute malaria were included in the study. When categorized according to the case definitions shown in Table 1, 38 (7.7%) had uncomplicated malaria (UM), 155 (31.4%) had mild MA (MlMA), 141 (28.6%) had moderate MA (MdMA), and 159 (32.3%) had SMA. Demographic and clinical characteristics of the study participants are shown in Table 2. There were significant differences in age (months) and Hb concentrations (g/dL) between the groups ($P < 0.001$ and $P < 0.001$, respectively, Table 2). The proportion of males versus females, parasite density (parasites/μL), and the proportion of children with HDP (≥ 10,000 parasites/μL) were not significantly different between the groups ($P = 0.08$, $P = 0.59$, and $P = 0.87$, respectively, Table 2).

Distribution of FcγRIIa genotypes among disease categories. The prevalences of FcγRIIa-131 genotypes in the population were 26.7% (132 of 493), 47.0% (231 of 493), and 26.3% (130 of 493) for H/H131, H/R131, and R/R131, respectively, with near equal allelic distribution ($p = 0.502$ and $q = 0.498$ for the H and R alleles, respectively). The prevalence of

TABLE 1
Definitions of disease categories*

Categories	Case definitions
Uncomplicated malaria	Children with a positive smear for *Plasmodium falciparum* parasitemia (of any density), absence of anemia (i.e., Hb > 11.0 g/dL), and free from the symptoms of severe malaria, such as hypoglycemia
Mild malarial anemia	Children with a positive smear for *P. falciparum* parasitemia (of any density), Hb = 8.0–10.9 g/dL, and free from the symptoms of severe malaria, such as hypoglycemia
Moderate malarial anemia	Children with a positive smear of *P. falciparum* parasitemia (of any density), Hb = 6.0–7.9 g/dL, and free from the symptoms of severe malaria such as hypoglycemia
Severe malarial anemia	Children with a positive smear for asexual *P. falciparum* parasitemia (of any density) and Hb < 6.0 g/dL. Children with cerebral malaria, a rare occurrence in the study area, were excluded from the study

* Hb = hemoglobin.

TABLE 2
Demographic and clinical characteristics of study participants*

Characteristic	UM	M/MA	MdMA	SMA	*P*
No. of subjects	38	155	141	159	
Age, months	15.16 (1.44)	12.49 (0.54)	11.25 (0.51)	9.79 (0.49)	< 0.001†
Sex					
Male, no. (%)	17 (44.7)	92 (59.3)	64 (45.4)	83 (52.2)	0.08‡
Female, no. (%)	21 (55.3)	63 (40.7)	77 (54.6)	76 (47.8)	
Hemoglobin, g/dL	11.65 (0.13)	9.35 (0.07)	6.86 (0.05)	4.79 (0.06)	< 0.001†
Parasitemia/μL	41,621 (6,123)	38,314 (3,979)	33,650 (3,219)	44,779 (4,913)	0.59‡
Geomean parasitemia/μL	18,661	13,723	14,277	14,875	
High-density parasitemia					
≥ 10,000 parasites/μL, no. (%)	27 (71.0)	103 (66.4)	93 (65.9)	102 (64.1)	0.87‡

* Children (n = 493) were categorized based on hemoglobin levels and presence of paratemia for the different disease categories. Data are presented as the mean (SEM) unless otherwise noted. UM = uncomplicated malaria; M/MA = mild malarial anemia; MdMA = moderate malarial anemia; SMA = severe malarial anemia.
† Statistical significance determined by the Kruskal-Wallis test.
‡ Statistical significance determined by the Chi-square analysis.

the FcγRIIa-131 genotypes was not significantly different between the groups ($P = 0.64$, Table 3). Since the UM group is representative of a favorable outcome during acute malaria, they were used as the reference group for comparing the frequency of the different genotypes between the groups. The frequency of the R/R131 genotype was lower in the UM group (21.1%) relative to the SMA (29.6%; $P = 0.29$) and MlMA (28.4%; $P = 0.36$) groups and equal to the MdMA group (22.0%; $P = 0.90$, Table 3). There was a lower frequency of heterozygosity for the 131 allele in the UM group (44.7%) compared with the MdMA group (51.8%; $P = 0.44$, Table 3). The frequency of H/R131 heterozygotes in the UM group (44.7%) was similar to that in the SMA (44.0%; $P = 0.41$) and M*l*MA groups (45.8%; $P = 0.99$, Table 3). The frequency of the H/H131 genotype was higher in UM group (34.2%) relative to the SMA (26.4%; $P = 0.33$), MdMA (26.2%; $P = 0.33$), and MlMA groups (25.8%; $P = 0.29$, Table 3).

Association of FcγRIIa genotypes with malaria disease severity. Multivariate logistic regression was used to determine the effect of FcγRIIa-131 variation on susceptibility to SMA, MA, and HDP. The confounding effects of age, sex, and sickle-cell trait (HbAS) were controlled for in the analyses because these variables significantly influence susceptibility to malaria. The multivariate model showed that relative to H/R131, the R/R131 and H/H131 genotypes were not associated with protection against SMA (odds ratio [OR] = 1.22, 95% confidence interval [CI] = 0.77–1.96, $P = 0.39$ and OR = 0.97, 95% CI = 0.60–1.56, $P = 0.85$, respectively, Table 4). Additional analyses based on the World Health Organization classification of SMA (Hb < 5.0 g/dL[17]) showed that there was no association between the FcγRIIa-131 genotypes and SMA. Relative to the H/R131 genotype, there was a non-significant protective effect against MA associated with the R/R131 (OR = 0.84, 95% CI = 0.53–1.33, $P = 0.46$) and H/H131 genotypes (OR = 0.81, 95% CI = 0.51–1.27, $P = 0.35$, Table 4). Additional analyses examining the relationship between FcγRIIa-131 variation and HDP demonstrated that relative to the H/R131 genotype, homozygous R131 alleles were significantly associated with protection against HDP (OR = 0.58, 95% CI = 0.37–0.92, $P = 0.02$), while homozygous H131 alleles were mildly protective against HDP (OR = 0.71, 95% CI = 0.45–1.13, $P = 0.14$, Table 4).

TABLE 3
Distribution of FcγRIIa-131 genotypes in the different disease categories*

FcγRIIa-131 genotype	UM	M/MA	MdMA	SMA	*P*
R/R131	8 (21.1)	44 (28.4)	31 (22.0)	47 (29.6)	0.64
H/R131	17 (44.7)	71 (45.8)	73 (51.8)	70 (44.0)	
H/H131	13 (34.2)	40 (25.8)	37 (26.2)	42 (26.4)	

* Data are presented as no. (%) for the FcγRIIa-131 genotypes in each of the disease categories for the study participants (n = 493). Statistical significance was determined by chi-square analysis. For definitions of abbreviations, see Table 1.

Since our recent investigations showed that pediatric HIV-1 infection significantly enhances severe anemia during acute malaria,[18] additional analyses were performed to determine if HIV-1 status impacted on our current findings. When controlling for HIV-1 infection, along with age, sex, and sickle-cell trait, relative to the H/R131 genotype, homozygous R131 alleles remained non-significantly associated with SMA (OR = 1.23, 95% CI = 0.77–1.98; $P = 0.38$), MA (OR = 0.84, 95% CI = 0.53–1.34, $P = 0.46$), but significantly protective against HDP (OR = 0.58, 95% CI = 0.37–0.92, $P = 0.02$). Additionally, when controlling for HIV-1 infection, results for the homozygous H131 alleles remained consistent for the risk of developing SMA (OR = 0.99, 95% CI = 0.61–1.60, $P = 0.95$), MA (OR = 0.82, 95% CI = 0.51–1.29, $P = 0.38$), and HDP (OR = 0.71, 95% CI = 0.44–1.12, $P = 0.14$).

DISCUSSION

Results presented here demonstrate that the R/R131 genotype is significantly associated with protection against HDP and that homozygous H131 alleles were mildly protective against HDP. These results are similar to previous studies in an adjacent area of western Kenya in which the R/R131 genotype was associated with protection against a lower threshold of parasitemia (> 5,000 parasites/μL) during the first year of life.[5] Although the association between FcγRIIa-131 variation and MA has not been reported for holoendemic *P. falciparum* transmission areas, results presented here show that the R/R131 and H/H131 genotypes were non-significantly associated with a decrease in the risk of MA (Hb < 8.0 g/dL), but not with SMA (Hb < 6.0 g/dL). These results may reflect that parasite density and the degree of anemia are not strongly associated in infants and young children with acute malaria in this geographic location.

In a large cohort in The Gambia, cross-sectional analyses showed that the H/H131 genotype was associated with increased malaria disease severity, particularly in children less

TABLE 4
Association of FcγRIIa-131 genotypes with malaria disease severity*

FcγRIIa genotypes	SMA (Hb < 6.0 g/dL)			MA (Hb < 8.0 g/dL)			HDP (≥ 10,000 parasites/μL)		
	OR	95% CI	*P*	OR	95% CI	*P*	OR	95% CI	*P*
H/R131	1.00			1.00			1.00		
R/R131	1.22	0.77–1.96	0.39	0.84	0.53–1.33	0.46	0.58	0.37–0.92	0.02
H/H131	0.97	0.60–1.56	0.85	0.81	0.51–1.27	0.35	0.71	0.45–1.13	0.15

* Children (n = 493) were categorized into three groups to examine the association between FcγRIIa-131 variants and malaria disease outcomes: severe malarial anemia (SMA, hemoglobin [Hb] < 6.0 g/dL with any density parasitemia), malarial anemia (MA, Hb < 8.0 g/dL, with any density parasitemia), or high-density parasitemia (HDP, ≥ 10,000 parasites/μL). Odds ratios (ORs) and 95% confidence intervals (CIs) were determined using logistic regression controlling for age, sex, and prevalence of sickle cell trait (HbAS), with the FcγRII-HR131 genotype as the reference. Additional analyses controlling for human immunodefiency virus type 1 status did not significantly alter the association of FcγRIIa-131 genotypes with any of the malaria disease outcomes presented.

than five years of age.[7] Although severe malaria in this population was defined by a mixed phenotype of disease (CM, SMA, and/or hypoglycemia), the H/H131 genotype was nonsignificantly more prevalent in those with SMA compared with aparasitemic children.[7] Another cross-sectional study in Thai adults showed that the H/H131 genotype, together with the FcγRIIIB-NA2 allele, was associated with increased susceptibility to CM.[8] Differences in results presented here showing that homozygosity for the H131 allele was nonsignificantly protective against HDP and MA versus previous findings likely reflect diverse immunoprotective responses to malaria conditioned by different exposure rates (i.e., hyperendemic versus holoendemic). In addition, since CM is a rare clinical manifestation of severe malaria in the study cohort and homozygous H131 alleles apparently increase susceptibility to CM, the effect of the H/H131 genotype may not be observed in a population in which the primary disease outcome of severe malaria is MA.

Previous findings showed that relative to H/R131 genotype, 1) homozygous H131 alleles are associated with increased HIV-1 prevalence in children born to HIV-positive mothers,[19] and 2) the H/H131 genotype is correlated with increased prevalence of placental malaria in HIV-positive women.[20] However, results presented here demonstrate that HIV-1 infection did not impact on the association of the FcγRIIa-131 genotypes with SMA, MA, and HDP.

The protective role of R/R131 against HDP may be due to the preferential binding of cytophilic IgG1 and IgG3 subtypes, which have been shown to mediate opsonization of parasitized red blood cells *in vitro* and enhance phagocytosis of these cells by monocyte/macrophages.[21] Furthermore, previous *in vitro* studies demonstrated that cytophilic IgG_1 and IgG_3 subtypes, as opposed to IgG_2 and IgG_4, provide protective immunity against malaria through the ability of monocytes to mediate antibody-dependent cellular inhibition (ADCI) of asexual blood stage merozoites.[22] However, it is unclear if ADCI can affect merozoite clearance *in vivo* because merozoites rapidly invade red blood cells and additionally, schizonts surrounded by rosettes of red blood cells may be protected against ADCI. In the context of findings presented here, which showed a protective effect against HDP and non-significant protection against MA, the selectivity of the R/R131 genotype for binding to IgG_1 and IgG_3 suggests that homozygous R131 alleles may increase parasite clearance, but may not be highly protective against MA in infants and young children who are largely malaria-naive. These results are consistent with the fact that both parasite density and HDP are relatively equivalent in the different disease categories, even though the degree of anemia during acute malaria is markedly different between the groups. Since MA is multifactorial and is influenced by nutritional deficiencies, hemoglobinopathies, and co-infection with other anemia-promoting pathogens, it is not unexpected that genetic variation can be associated with protection against HDP, but not MA. By following the children longitudinally and determining the co-factors responsible for anemia, it should be possible to determine the effect of FcγRIIa-131 polymorphic variants on susceptibility to MA as the children acquire protective malarial immunity during the first three years of life. The fact that homozygous R131 alleles protect against HDP, but not MA, the primary cause of morbidity and mortality in holoendemic *P. falciparum* transmission areas, underscores the complexity associated with determining critical host immune response genes that protect against severe disease outcomes.

In addition to IgG_1 and IgG_3, protective immunity against malaria may also involve IgG_4, as shown by studies demonstrating a significant decrease in the level of IgG_3 and enhancement of IgG_4 anti–variant surface antigens (VSA) responses in aparasitemic malaria-exposed children.[23] In those studies, clinical protection and prolonged intervals between malaria parasitemia were associated with high levels of IgG_4 and antibodies to VSA IgG_1 in severe and mild *P. falciparum* malaria, respectively. In results presented here, the H/H131 genotype that binds efficiently to IgG_2, as well as IgG_1 and IgG_3,[4] was associated with mild protection against HDP. This is in agreement with previous observations that IgG_2 antigen-specific responses may correlate with disease protection, even though these antibodies appear less protective than IgG_1 and IgG_3.[24] Additional studies are required to resolve the role of IgG_2 in mediating protective immunity against malaria and the impact of FcγRIIa polymorphic variants on conditioning disease outcomes.

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