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ORIGINAL ARTICLE

A macrophage migration inhibitory factor promoter polymorphism is associated with high-density parasitemia in children with malaria

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Macrophage migration inhibitory factor (MIF) is a pleiotropic cytokine that regulates innate and adaptive immune responses to bacterial and parasitic infections. Functional promoter variants in the MIF gene influence susceptibility to inflammatory diseases in Caucasians. As the role of genetic variation in the MIF gene in conditioning malaria disease outcomes is largely unexplored, the relationship between a G to C transition at MIF −173 and susceptibility to high-density parasitemia (HDP) and severe malarial anemia (SMA) was examined in Kenyan children (aged 3–36 months; n = 477) in a holoendemic Plasmodium falciparum transmission region. In a multivariate model, controlling for age, gender, HIV-1 status, and sickle-cell trait, MIF −173CC was associated with an increased risk of HDP compared to MIF −173GG. No significant associations were found between MIF −173 genotypic variants and susceptibility to SMA. Additional studies demonstrated that homozygous G alleles were associated with lower basal circulating MIF levels relative to the GC group. However, stimulation of cultured peripheral blood mononuclear cells with malarial pigment (hemozoin) increased MIF production in the GG group and decreased MIF production in the GC group. Thus, variability at MIF −173 is associated with functional changes in MIF production and susceptibility to HDP in children with malaria.
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Introduction

Malaria is one of the leading causes of childhood morbidity and mortality in sub-Saharan Africa, accounting for 25–35% of the outpatient visits, 20–45% of the hospital admissions, and up to 35% of inpatient deaths.[1] The vast majority of the global malaria cases occur in sub-Saharan Africa in which greater than 90% of the clinical cases are caused by *Plasmodium falciparum* infections.[1] Clinical manifestations of *P. falciparum* malaria vary widely, and range from mild fevers to severe life-threatening complications including hyperparasitemia, hypoglycemia, renal insufficiency, cerebral malaria (CM), severe malarial anemia (SMA), and respiratory distress.[2–4]

Transmission intensity and the age at which malaria is acquired are important determinants of the clinical manifestations of the disease.[5] However, transmission intensity and age do not adequately explain variation in malaria disease severity among age-matched infants and young children (aged 0–3 years) with similar levels of parasite exposure and infection rates. Diverse clinical outcomes under these circumstances appear to be conditioned by genetic variability as malaria has exerted significant selective pressure on the human genome, particularly in host-immune response genes that mediate susceptibility and clinical outcomes of the disease.[6]

Macrophage migration inhibitory factor (MIF) is a ubiquitous cytokine produced by T cells,[7,8] monocytes/macrophages,[9] and the anterior pituitary gland[10] in response to proinflammatory stimuli. Unlike most cytokines, MIF is constitutively expressed at high levels and stored in preformed vesicles, and therefore, can be rapidly released without *de novo* gene expression.[10,11] MIF has potent proinflammatory properties and is an important mediator of both innate and adaptive immune responses to bacterial and parasitic infections.[8,12–16]

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Variation in the MIF gene has been shown to influence susceptibility to several inflammatory diseases in non-African populations, including rheumatoid arthritis, atopy, ulcerative colitis, and lung disease.[17–19] To date, five polymorphisms have been identified in the MIF gene, four single-nucleotide polymorphisms (SNPs) at positions −173 (G/C), +24 (A/T), +254 (T/C) and +656 (C/G), and a tetranucleotide repeat at −794 ($CATT_{5-8}$).[20–22] However, only the MIF −173 and MIF −794 polymorphisms have been reported to affect both basal and stimuli-induced MIF production, and influence susceptibility to chronic inflammatory and infectious diseases in Caucasians.[17,20–25] In addition, high MIF-producing alleles of the −794 CATT repeat were associated with increased susceptibility to high-density parasitemia (HDP, ⩾10 000 parasites/μl) in Zambian children with acute malaria.[26] The role of polymorphic variability in MIF −173 in influencing susceptibility to severe malaria, however, has not been elucidated.

Although elevated MIF levels are associated with enhanced pathogenesis in murine models of malaria,[27,28] investigations in human malaria have yielded contrasting findings.[28,29] Previous investigations showed that MIF production was elevated in intervillous blood during placental malaria,[30,31] thoracic blood vessels of Malawian children with CM,[32] and in peripheral blood from Zambian children with acute malaria.[28] However, we have recently shown that circulating MIF concentrations and peripheral blood mononuclear cells (PBMC) MIF transcripts are suppressed in Gabonese children with mild-to-moderate forms of malarial anemia and hyperparasitemia,[29] and in Kenyan children with SMA (Awandare *et al*., unpublished observations).

To further define the role of MIF in the immunopathogenesis of malaria, we investigated the impact of polymorphic variability at MIF −173 on susceptibility and clinical outcomes of severe malaria, and MIF production. To accomplish these experimental objectives, we performed a cross-sectional, case–control study in a large population of infants and young children with acute malaria (cases) and healthy, aparasitemic individuals (controls). Results presented here describe the relationship between MIF −173 variants and susceptibility to HDP (⩾10 000 parasites/μl) and SMA (hemoglobin (Hb) < 6.0 g/dl). In addition, we describe the functional association between MIF −173 genotypes and circulating MIF levels in children with and without malaria, and MIF production in cultured peripheral blood mononuclear cells (PBMC) stimulated with malarial pigment (hemozoin, *pf*Hz).

Results

Clinical and parasitological characteristics of study participants

Previous studies in Zambian children illustrate that variability at MIF −794 is associated with parasitemic outcomes in children with acute malaria.[26] To investigate the role of variability at MIF −173 in influencing susceptibility and outcomes of parasitemia, children ($n = 477$; age, 3–36 months) presenting at a rural hospital with acute malaria or for routine immunizations were stratified according to parasite density: aparasitemic controls (AC, $n = 114$), low-density parasitemia (LDP, <10 000 parasites/μl; $n = 127$), and high-density parasitemia (HDP, ⩾10 000 parasites/μl; $n = 236$). The clinical and parasitological characteristics of the study participants upon admission are summarized in Table 1. There were no significant differences in gender distribution among the groups ($P = 0.687$). Age was significantly different across the groups ($P < 0.05$), largely because children in the AC group were significantly younger than those with HDP ($P < 0.01$); the differences in age between the LDP and AC ($P = 0.144$) or HDP ($P = 0.236$) groups were not significant. Axillary temperature differed across the groups ($P < 0.0001$), with children in the HDP group having significantly higher temperatures than those with LDP ($P < 0.005$). In addition, Hb concentrations were significantly different across the three groups ($P < 0.001$). Children with LDP had lower Hb levels than the HDP group; however,

Table 1 Demographic, parasitological, and hematological characteristics of study participants

Characteristic	*AC*	*LDP*	*HDP*	P
Number (*n*)	114	127	236	
Gender (n, %)				
Female	57 (50)	60 (47)	120 (51)	0.687[a]
Male	57 (50)	67 (53)	116 (49)	
Age (months)	10.6 (0.8)	11.0 (0.5)	11.5 (0.4)[b]	0.033[c]
Axillary temperature (°C)	37.1 (0.1)	37.3 (0.1)	37.8 (0.1)	0.0001[c]
Parasitemia (/μl)	0	3584 (222)	56 652 (2869)	<0.0001[d]
Geomean parasitemia (/μl)	0	1998	39 756	<0.0001[e]
Hemoglobin (g/dl)	9.9 (0.2)	6.8 (0.2)	7.2 (0.1)[b]	<0.001[c]
SMA (*n*, %)	NA	47 (37.4)	84 (35.5)	0.669[a]

Abbreviations: AC, aparasitemic controls (*P. falciparum*-negative); LDP, low-density parasitemia (<10 000 parasites/μl); HDP, high-density parasitemia (⩾10 000 parasites/μl); SMA, severe malarial anemia (Hb < 6.0 g/dl); NA, not applicable.
Data are presented as mean (s.e.m.) except otherwise indicated.
[a]Chi-square test.
[b]Not significantly different from LDP group.
[c]Kruskal–Wallis test.
[d]Mann–Whitney *U*-test for HDP vs LDP.
[e]Student's *t* test for HDP vs LDP.

these differences did not reach statistical significance ($P = 0.061$). Despite the large disparity in parasite densities between LDP and HDP groups, the proportions of children with SMA (Hb < 6.0 g/dl) in these two groups were not significantly different ($P = 0.669$). These results illustrate that concomitant peripheral parasite density and SMA are largely independent in children presenting at hospital in this holoendemic area of *P. falciparum* transmission.

Distribution of MIF −173 genotypes

The genotypic distribution of the MIF −173G/C polymorphism in AC ($n = 114$) and children with acute malaria ($n = 363$) is shown in Table 2. In the 477 children examined, 19% were GG, 43% were GC, and 38% were CC, representing a significant departure from Hardy–Weinberg equilibrium (HWE; $\chi^2 = 6.01$, $P < 0.01$). Proportions of children with malaria from each genotypic group were 80% GG, 78% GC, and 72% CC. The genotypic distribution in AC was 16, 38, and 46% for the GG, GC, and CC, respectively. Frequencies of the G and C alleles were 0.35 and 0.65 in AC with no departure from HWE ($\chi^2 = 2.70$, $P = 0.10$). Among children with acute malaria, there were 20% GG, 44% GC, and 36% CC yielding G and C allele frequencies of 0.42 and 0.58, respectively. There was no significant evidence of departure from HWE ($\chi^2 = 3.29$, $P = 0.075$). χ^2 analysis revealed that there was also no significant difference in the frequency distribution of the MIF −173G/C polymorphism in cases compared to controls ($\chi^2 = 2.10$, $P = 0.349$).

Table 2 Genotypic distribution of the MIF −173G/C polymorphism

MIF −173 genotype	*Aparasitemic controls* n *(%)*	*Malaria cases* n *(%)*	*Total* n *(%)*
GG	18 (16)	72 (20)	90 (19)
GC	44 (38)	160 (44)	204 (43)
CC	52 (46)	131 (36)	183 (38)
	$n = 114$	$n = 363$	$n = 477$
	P(G) = 0.35	P(G) = 0.42	P(G) = 0.40

Abbreviations: MIF, macrophage migration inhibitory factor; P(G), frequency of G allele.

Association of MIF −173 genotypic variants with malaria disease outcomes

The association between variation at MIF −173 and malaria disease severity was determined by multivariate logistic regression analyses. Parasitemia (*P. falciparum*-positive blood smear), HDP, and SMA were the primary disease outcomes, controlling for age, gender, and sickle-cell status. As our recent studies also demonstrate that both HIV-1 exposure and HIV-1 virus increase the risk of developing SMA in the current study cohort,[33] HIV-1 status was also controlled for in the analyses. Relative to homozygous G alleles, the GC and CC genotypes were 10% ($P = 0.861$) and 30% ($P = 0.257$) less likely to have parasitemia, respectively (Table 3). However, among parasitemic children, the GC and CC genotypes were associated with a 70% ($P = 0.065$) and 90% ($P = 0.039$) increased risk of developing HDP, respectively, relative to the GG group (Figure 1 and Table 3). Analyses of the relationship between the MIF −173 polymorphism and SMA (Hb < 6.0 g/dl) revealed that children in the GC group had a 30% ($P = 0.307$) reduced risk of developing

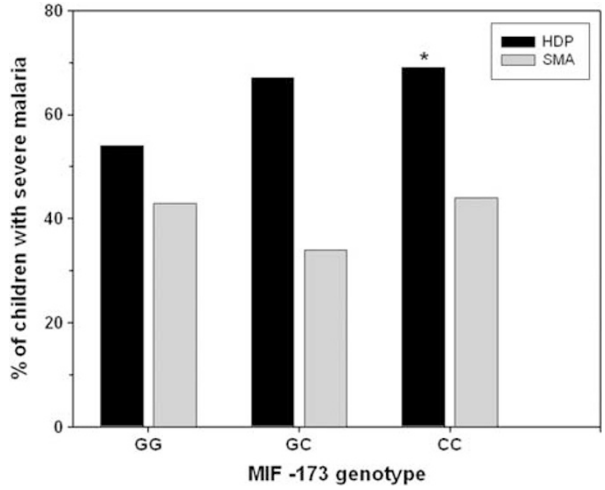

Figure 1 Proportion of HDP and SMA stratified according to MIF −173G/C genotype. Proportion of malaria cases with HDP (⩾10 000 parasites/μl) and SMA (Hb < 6.0 g/dl) are presented for each MIF −173 genotypic category (GG, $n = 72$; GC, $n = 160$; CC, $n = 130$). *Significantly higher compared to the GG group ($P < 0.05$), χ^2 test.

Table 3 Association of MIF −173G/C polymorphism with disease susceptibility and severity

MIF −173 genotype	*Parasitemia (P. falciparum +)*			*HDP (⩾10 000 parasites/μl)*			*SMA (Hb < 6.0 g/dl)*		
	OR	*95% CI*	P	*OR*	*95% CI*	P	*OR*	*95% CI*	P
GG	1.0			1.0			1.0		
GC	0.9	0.5–1.8	0.861	1.7	1.0–3.1	0.065	0.7	0.4–1.3	0.307
CC	0.7	0.4–1.3	0.257	1.9	1.1–3.5	**0.039**	1.0	0.6–1.9	0.960

Abbreviations: CI, confidence interval; HDP, high-density parasitemia; MIF, macrophage migration inhibitory factor; OR, odds ratio; SMA, severe malarial anemia.

Statistically significant *P*-value is in bold.

Data presented are results of multivariate logistic regression analyses controlling for age, gender, HIV-1 status, and sickle-cell status.

Association between MIF −173 genotypes and susceptibility to malaria infection (*P. falciparum*-positive blood smear) was examined in 477 children consisting of 114 aparasitemic controls and 363 malaria cases. Analyses of relationships between MIF −173 genotypes and high-density parasitemia (HDP, ⩾10 000 parasites/μl), and severe malarial anemia (SMA, Hb < 6.0 g/dl) were performed in parasitemic children only ($n = 363$). The GG genotype was used as reference for these analyses, as this genotype was considered wild type in previous studies.[20,21]

SMA compared to those with the GG genotype, whereas homozygous C alleles had no impact on the development of SMA in parasitemic children ($P = 0.960$; Figure 1 and Table 3). Additional analyses conducted using the WHO definition of SMA (i.e., Hb < 5.0 g/dl)[34] also failed to yield any significant associations between MIF −173G/C polymorphism and SMA (GC vs GG, $P = 0.707$ and CC vs GG, $P = 0.967$). Taken together, these findings illustrate that the MIF −173G/C polymorphism is associated with increased susceptibility to HDP, but not SMA, consistent with the data presented above (Table 1) demonstrating that parasite density and anemia severity are not significantly associated in this holoendemic *P. falciparum* transmission area.

Functional relationship between MIF −173G/C polymorphism and circulating MIF levels

To examine the functional relationship between the polymorphism and plasma MIF concentrations, AC ($n = 114$) and children with acute malaria ($n = 363$) were analyzed separately, as the presence of parasitemia can alter circulating MIF levels.[29] Among AC, plasma MIF levels were significantly different across the genotypic groups ($P < 0.05$, Figure 2). Relative to homozygous G alleles (median (interquartile range), 2179 (1452–9341) pg/ml), median circulating MIF concentration was 1.9 times higher in the GC group (4145 (2822–6288) pg/ml, $P < 0.05$) and 1.7 times elevated in the CC group (3701 (2142–5747) pg/ml, $P = 0.322$; Figure 2). However, peripheral blood MIF concentrations in children with acute malaria were not significantly different across the genotypic categories (GG, 4347 (2421–7199) pg/ml; GC, 3915 (2045–5930) pg/ml; CC, 4085 (2704–5936) pg/ml; $P = 0.291$, Figure 2).

Influence of MIF −173G/C polymorphism on MIF production in pf*Hz-stimulated PBMC*

Several studies from our laboratory and others have demonstrated that phagocytosis of *pf*Hz is associated with cytokine, chemokine, and effector molecule dysregulation *in vivo*,[35–37] and stimulation of macrophages or PBMC with *pf*Hz *in vitro* elicits a cytokine/chemokine/effector molecule production profile similar to that observed during malaria infection.[28,38–44] Therefore, to further examine the functional significance of variation at MIF −173, PBMC were cultured from healthy malaria-naïve US individuals with differing genotypes and stimulated with *pf*Hz. As shown in Figure 3, stimulation with physiological concentrations of *pf*Hz[39] significantly increased MIF production in individuals with homozygous G alleles ($P < 0.05$), whereas treatment with *pf*Hz significantly decreased MIF production in heterozygous individuals ($P < 0.05$, Figure 3). Individuals with homozygous C alleles were not available for these analyses. Taken together, these results demonstrate that variation at MIF −173 is associated with differential MIF production in response to malaria parasite products.

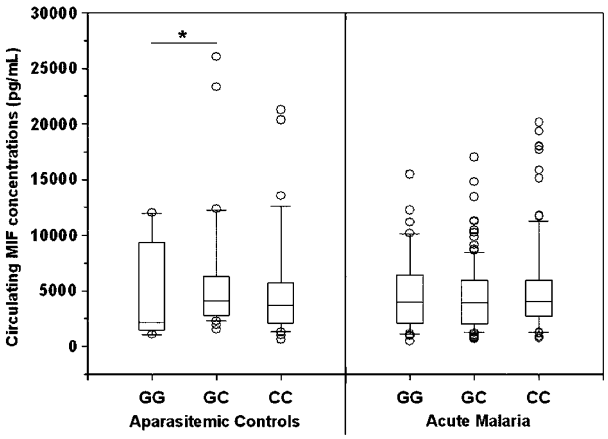

Figure 2 Circulating MIF levels in the MIF −173G/C genotypic categories. Plasma levels of MIF in AC (GG, $n = 14$; GC, $n = 32$; CC, $n = 35$) and malaria cases (GG, $n = 50$; GC, $n = 124$; CC, $n = 102$) were measured by enzyme-linked immunosorbent assay (ELISA) and are presented according to MIF −173 genotype. Boxes represent the interquartile range, the line through the box represents the median, whiskers illustrate the 10th and 90th percentiles, and symbols represent outliers. *Differences between groups were statistically significant by Mann–Whitney *U*-test ($P < 0.05$).

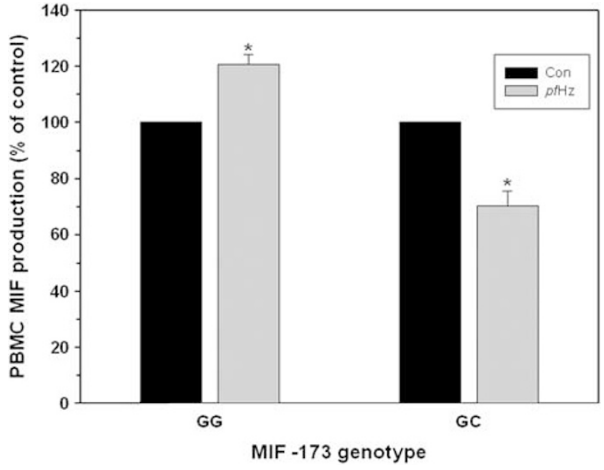

Figure 3 MIF production in *pf*Hz-stimulated PBMC in the MIF −173G/C genotypic categories. PBMC obtained from healthy, US donors with the GG ($n = 3$) and GC ($n = 3$) genotypes at MIF −173 were stimulated with media alone (Con) or a physiological concentration of *pf*Hz (10 μg/ml). MIF concentrations were determined by ELISA in culture supernatants after 48 h of incubation and are expressed as percent of Con. Data are presented as mean (SEM) for $n = 3$ donors per genotypic group. *$P < 0.05$ compared to Con, Student's *t*-test.

Discussion

This study presents the first report on the association between the MIF −173G/C polymorphism and susceptibility to severe malaria. Distribution of the MIF −173 polymorphism in the Kenyan cohort examined here parallels studies in Zambian children showing a higher frequency of the C allele.[26] Distribution of the C and G alleles, therefore, differs substantially between sub-Saharan African ethnic groups and Caucasian populations in which the G allele is more prevalent.[20,21] Differences in allelic frequencies across populations may be owing to selective pressure from infectious diseases, such as malaria, that have historically occurred in certain climates and not in others. Consistent with a role of MIF in conditioning outcomes to infectious

diseases,[8,12–16] multivariate modeling revealed that the CC genotype was significantly associated with an increased risk of HDP, whereas heterozygosity was associated with a moderately higher risk of developing HDP. However, there was no association between MIF −173 genotypes and SMA, supporting the observation that malarial anemia and parasite burden are not significantly related in this cohort of children. Results presented here showing that parasitemia and the severity of malarial anemia are largely unrelated upon presentation at hospital are consistent with previous studies showing that parasite density during the preceding 3 months, rather than concomitant parasitemia, predicts the risk of developing childhood SMA in western Kenya.[45]

The G to C transition at MIF −173 creates a potential transcription factor-binding site for activator protein (AP)-4, suggesting that polymorphic variability at −173 could functionally alter MIF production.[21] Cloning of a portion of the MIF gene (−775 to +84; excluding the CATT repeat at −794) into a luciferase reporter vector demonstrated that the −173C promoter was more active in CEM C7A (lung epithelial) cells, whereas the −173G promoter had the highest activity in A549 (T lymphoblast) cells.[21] These results illustrate the complex relationship between MIF promoter variants and regulation of MIF production. Examination of the functional association between variability at MIF −173 and circulating MIF levels revealed that the C allele was correlated with increased peripheral blood MIF concentrations in AC. These results parallel previous studies in individuals with chronic inflammatory diseases in which the C allele was associated with increased serum MIF concentrations.[21,23] Circulating MIF levels, however, were not significantly different across the genotypic groups with acute malaria. Of interest, when MIF levels were compared between AC and acute malaria cases (Figure 2), homozygous G alleles were associated with a nearly twofold increase in MIF levels in parasitemic children, whereas the GC and CC genotypes had similar MIF concentrations in cases and controls. We hypothesize that despite lower baseline MIF production in the GG group, their ability to mount a potent MIF response may aid in controlling parasitemia. This hypothesis is supported by the finding that homozygous G alleles were associated with a decreased risk of developing HDP.

We have previously observed that stimulation of PBMC with *pf*Hz or synthetic hemozoin (sHz) (Awandare *et al*., unpublished observations) suppresses MIF production, whereas others have demonstrated that sHz increases PBMC MIF production with specific variants of the MIF −794 polymorphism (5-CATT/5-CATT, 6-CATT/6-CATT, and 6-CATT/7-CATT).[28] However, the influence of variation in the MIF gene on MIF production was not determined in our previous studies. Data presented here demonstrate a dichotomous pattern of MIF responses in PBMC stimulated with *pf*Hz; GG individuals had increased MIF production, whereas GC individuals had decreased MIF production, suggesting that the pattern of MIF production during malaria is largely influenced by variation at MIF −173. Identical results were obtained using sHz (data not shown), suggesting that the core ferriprotoporphorin IX structure of hemozoin is responsible for altering MIF production, rather than adherent host or parasite-derived proteins, lipids, or nucleic acids. It remains to be determined how individuals with homozygous C alleles at MIF −173 respond to challenge with malarial pigment as these individuals were not available for analyses, largely because of the low frequency of this genotype in Caucasian populations.[20,21]

Several studies have demonstrated linkage disequilibrium between the MIF −173 SNP and the upstream MIF −794 CATT repeat polymorphism, with haplotypes of the two polymorphic sites being strongly associated with functional gene expression and susceptibility to inflammatory disease.[18,21,23] Therefore, although not examined in this study, it is possible that some of the relationships between the MIF −173 SNP and malaria disease severity, as well as MIF production may be influenced by the upstream CATT repeat polymorphism. Previous results in reporter constructs, however, demonstrate that variation at −173 alters MIF production in the absence of the −794 CATT polymorphism,[21] suggesting that effects of variation at the two sites may be, at least in part, independent.

Taken together, results presented here illustrate that variation at MIF −173 is associated with functional differences in MIF production and susceptibility to severe malaria. These data further illustrate that MIF −173 variants that confer protection against HDP are also associated with increased MIF production in response to stimulation by malaria parasite products (*pf*Hz). Given the critical role of MIF in mediating protective immune responses to other infections, including *Salmonella typhii*[15] and *Leishmania major*,[16] a potent MIF response may be required for effective control of parasitemia during malaria. As recent studies illustrate that variation in the MIF −794 tetranucleotide repeat is associated with susceptibility to HDP in Zambian children,[26] we are currently examining the haplotypic distributions of MIF −173 and MIF −794 polymorphisms to obtain additional insight into the role of genetic variation in the MIF gene in conditioning malaria disease outcomes.

Study participants and methods

Study site

Study participants ($n = 477$) were recruited at the pediatric ward of the Siaya District Hospital (SDH), Nyanza Province, western Kenya. *P. falciparum* transmission in this region is holoendemic with entomological inoculation rates of 100–300 infective bites per annum.[46] Common clinical presentations of severe *P. falciparum* malaria at SDH include HDP and SMA, with CM occurring only in rare cases.[47,48] This area provides a homogenous population for investigating genetic associations with disease susceptibility, as >99% of the inhabitants belong to the Luo ethnic group.[48] Additional detail on the study location and manifestations of malaria in the study cohort are presented in our recent publication.[48]

Study participants

Study participants (aged 3–36 months) were enrolled after obtaining written, informed consent from the parents/guardians. The study was approved by the Ethics Committees of the Kenya Medical Research

Institute and the University of Pittsburgh Institutional Review Board. Malaria cases ($n=363$) were recruited from children presenting at SDH for their first hospital contact for the treatment of malaria. Children attending SDH for routine childhood immunizations, free of malaria parasites, afebrile and without history of diarrhea for at least 2 weeks were enrolled as healthy, AC ($n=114$). All children were from the Luo ethnic group. HDP was defined using 10 000 parasites/μl as cutoff as per previous studies from the same geographic location,[49] and elsewhere.[26] SMA was defined as Hb <6.0 g/dl with any parasite density based on previous investigations examining over 10 000 repeated Hb measurements in an age- and geographically matched cohort from the region of western Kenya where the present studies were conducted.[45] Only children infected with the *P. falciparum* species were included in this study; those with detectable *P. ovale* or *P. malariae* species were excluded from this study. None of the study participants had CM. Children with prior hospitalizations for any cause were excluded from the study.

Laboratory evaluation
Giemsa-stained thin and thick blood smears were used for determination of parasitemia. The number of asexual parasites per 300 leukocytes was obtained and parasites/μl were calculated as described previously.[50] Hb concentrations were determined using a Hemocue® system (Hemocue AB, Angelholm, Sweden). HIV-1 status was determined using two serological methods (Unigold™ (Trinity Biotech, Carlsbad, CA, USA) and Determine™ (Abbott Laboratories, Abbott Park, IL, USA)), and positive serological results were confirmed by proviral DNA polymerase chain reaction (PCR) as described previously.[33] All parents/guardians of the study participants received pre- and post-test HIV/AIDS counseling. None of the study participants were receiving antiretroviral drugs at the time of enrollment. Sickle-cell status was determined by alkaline cellulose acetate electrophoresis on Titan III plates (Helena BioSciences, UK) according to the manufacturer's recommendations.

Determination of plasma MIF
Before administration of antimalarials and/or any other treatment interventions, venous blood (<3 ml: a volume determined to be safe based on size, weight, and anemia status) was obtained from each study participant as described previously.[48] Concentrations of MIF in plasma and culture supernatants were determined using an enzyme-linked immunosorbent assay (ELISA) with a matched anti-MIF antibody pair (R&D systems, Minneapolis, MN, USA). All samples were assayed at 1:5 and 1:10 dilutions in duplicate, and assays were performed according to manufacturer's recommendations with the limit of detection >62.5 pg/ml.

Genotyping
Blood spots were collected on FTA Classic® cards (Whatman Inc., Clifton, NJ, USA) and stored at ambient temperature until DNA isolation. DNA was extracted using the Gentra System (Gentra System Inc., Minneapolis, MN, USA). The MIF-173G/C SNP was genotyped using a Taqman® 5′ allelic discrimination Assay-By-Design method (rs755622, Applied Biosystems, Foster City, CA, USA). The primer sequences were 5′-CGATTTCTAGCCGCCAAGTG-3′ (forward) and 5′-AGCAACCGCCGCTAAGC-3′ (reverse), whereas the Taqman 'minor groove binder' (MGB) probe sequences were (VIC)5′-AGAACAGGTTGGAGCG-3′ and (FAM)5′-AGAACAGCTTGGAGCG-3′. PCR was performed in a total volume of 5 μl with the following amplification protocol: 95°C for 10 min (95°C for 15 s, 60°C for 1 min) $\times 40$ cycles. Following PCR, the genotype of each individual was assigned by measuring allelic-specific fluorescence on the ABI Prism® 7900HT sequence detection system using the SDS 2.1® software for allelic discrimination (Applied Biosystems, Foster City, CA, USA). To validate results obtained with the Taqman® real-time genotyping assays, $\sim$10% of the samples were randomly selected and genotyped using restriction fragment length polymorphism (RFLP) PCR as described previously.[20] There was 100% concordance between the two methods for the samples tested using both methods.

PBMC cultures
PBMC were isolated from venous blood obtained from healthy, US donors using Ficoll-Hypaque as described previously.[51] To ensure complete removal of red blood cell (RBC), PBMC were treated with RBC lysis buffer (BioWhittaker, USA) for 5 min and then washed before culture. *pf*Hz was isolated from *P. falciparum* (PfD6) parasites cultivated on type O+ RBC as described in our previous report.[40] The *pf*Hz preparation was tested for the presence of endotoxin using Limulus amebocyte lysate test (LAL, BioWhittaker, Walkersville, MD, USA), and endotoxin levels were found to be <0.125 U/ml (i.e., <0.025 ng/ml). PBMC were plated at 1×10^6 cells/ml in Dulbecco's modified Eagle's medium (DMEM) containing *N*-2-hydroxyethylpiperazine-*N*′prime-2-ethanesulfonic acid (HEPES) buffer (25 mM), penicillin (100 U/ml)/streptomycin (100 μg/ml), and 10% heat inactivated human serum from a non-malarious region, and stimulated with media alone (unstimulated control) or a physiological concentration of *pf*Hz (10 μg/ml) as described previously.[39]

Statistical analyses
Kruskal–Wallis tests were used to compare variables across three or more groups, and where significant differences were observed, Mann–Whitney *U*-tests were conducted for pairwise comparisons. To determine associations between MIF -173 genotypes and disease severity, multivariate logistic regression analyses were conducted for each clinical definition (i.e., presence of parasitemia, HDP, and SMA) using a model that controlled for age, sex, HIV-1 status (which included both HIV-1 exposed and HIV-1 PCR(+) results), and sickle-cell trait. Statistical significance for all analyses was determined using a critical α-value of 0.05.

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Conflict of interest

There is no conflict of interest for any of the authors of the manuscript due to either commercial or other affiliations.

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