Functional Promoter Haplotypes of Interleukin-18 Condition Susceptibility to Severe Malarial Anemia and Childhood Mortality

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Severe malarial anemia (SMA) is a leading cause of morbidity and mortality in children residing in regions where Plasmodium falciparum transmission is holoendemic. Although largely unexplored in children with SMA, interleukin-18 (IL-18) is important for regulating innate and acquired immunity in inflammatory and infectious diseases. As such, we selected two functional single-nucleotide polymorphisms (SNPs) in the IL-18 promoter (−137G→C [rs187238] and −607C→A [rs1946518]) whose haplotypes encompass significant genetic variation due to the presence of strong linkage disequilibrium among these variants. The relationship between the genotypes/haplotypes, SMA (hemoglobin [Hb], <5.0 g/dl), and longitudinal clinical outcomes were then investigated in Kenyan children (n = 719). Multivariate logistic regression analyses controlling for age, gender, sickle cell trait, glucose-6-phosphate dehydrogenase (G6PD) deficiency, HIV-1, and bacteremia revealed that carriers of the −607AA genotype was associated with protection against SMA (odds ratio [OR] = 0.440 [95% confidence interval {CI} = 0.21 to 0.90], P = 0.031) in children with acute infection. In contrast, carriers of the −137G/−607C (GC) haplotype had increased susceptibility to SMA (OR = 2.050 [95% CI = 1.04 to 4.05], P = 0.039). Measurement of IL-18 gene expression in peripheral blood leukocytes demonstrated that elevated IL-18 transcripts were associated with reduced hemoglobin concentrations (ρ = −0.293, P = 0.010) and that carriers of the “susceptible” GC haplotype had elevated IL-18 transcripts (P = 0.026). Longitudinal investigation of clinical outcomes over a 3-year follow-up period revealed that carriers of the rare CC haplotype (~1% frequency) had 5.76 times higher mortality than noncarriers (P = 0.001). Results presented here demonstrate that IL-18 promoter haplotypes that condition elevated IL-18 gene products during acute infection are associated with increased risk of SMA. Furthermore, carriage of the rare CC haplotype significantly increases the risk of childhood mortality.

Malaria remains one of the most important parasitic infections in the world (49). Of the known apicomplexan Plasmodium parasites that can infect humans, P. falciparum, a species endemic to Africa, accounts for a vast majority of malaria-associated morbidity and mortality (10, 18, 49). Each year, malaria accounts for an estimated 247 million new cases, which result in approximately 881,000 deaths, 91% of which occur in Africa and 85% being in children under 5 years of age (50). In areas of high P. falciparum transmission, malaria infection manifests primarily as severe anemia, high-density parasitemia (HDP), respiratory distress, acute renal failure, and in rare cases, hypoglycemia and cerebral malaria (11, 25, 28, 41). The most common of these disease sequelae, severe malarial anemia (SMA), is responsible for the majority of the malaria-associated mortality in western Kenya (7, 32, 54). Based on historical presence of the disease, malaria has exerted a large impact on the human genome such that potentially harmful variants are preserved, largely because of the advantage offered in heterozygous individuals that are often protected from severe, complicated, and fatal malaria (14, 19, 21). Studies in our laboratory focused on variations in key cytokine genes have demonstrated associations between polymorphisms and SMA (4, 37, 38).

Interleukin-18 (IL-18) is a proinflammatory cytokine with diverse pleiotropic effects (30). Earlier studies designated IL-18 a gamma interferon (IFN-γ)-inducing factor due to its ability to induce production of IFN-γ from natural killer (NK) cells, T cells, and activated macrophages (31). IL-18 is synthesized as a precursor protein (proIL-18) and processed by an intracellular cysteine protease, caspase-1 (30). IL-18 is known to regulate both T helper 1 (Th1) and Th2 responses, depending on the cytokine milieu (31), and acts in vivo in synergy with...
IL-12 (33). Although IL-18 has a structure homologous to IL-1 and a significant functional homology to IL-12 in mediating Th1 responses and NK cell activity (30, 31), the mechanisms by which IL-18 induces IFN-γ seem to differ from those of IL-12 (46). Given its important role in the inflammatory process, IL-18 has extensively been studied in various disease pathologies, including digestive inflammatory diseases, human immunodeficiency virus (HIV) infection, diabetes, arthritis, asthma, tuberculosis, and cancer (27, 31).

A previous study in western Kenya investigating the relationship between IL-12 and IL-18 and also clinical malaria phenotypes in children (2 to 12 years of age) reported upregulation of IL-18 in uncomplicated malaria, which progressively declined in moderate malaria, and there was a further decrease in children with SMA (hemoglobin [Hb] <5.0 g/dl and any density parasitemia and fever) cases (8). These results parallel another study showing significantly elevated IL-12 and IL-18 in children (2 to 144 months of age) with mild malaria that decreased as disease severity progressed (24). In contrast, a study in adults (14 to 63 years of age), investigating the association between cytokine and antibody responses and uncomplicated, severe, and cerebral forms of malaria, demonstrated a close association between increased IL-18 levels and severe falciparum malaria (20, 29). These results are similar to an investigation showing elevated IL-18 in adult patients (mean age, 37.7 ± 5.9 years) with uncomplicated malaria, which decreased upon recovery of disease (44). Taken together, these results suggest that IL-18 plays an important role in conditioning severe malaria. However, no studies to date have reported the role of polymorphic variants of the IL-18 gene in modulating malaria.

Two IL-18 single-nucleotide polymorphisms (SNPs; −137G→C [rs187238] and −607C→A [rs1946518]) have consistently been associated with altered IL-18 transcriptional activity (13, 23, 53). The G-to-C substitution at position −137 abolishes a histone 4 transcription factor-1 (H4TF-1) nuclear factor-binding site, while the C-to-A transversion at position −607 disrupts a cyclic AMP (cAMP)-responsive element protein-binding site (13). Consequently, lower promoter activity has been reported for the minor alleles −137C and −607A, respectively (3, 23, 53). Furthermore, haplotypes carrying these minor alleles also correlate with decreased IL-18 levels in peripheral blood mononuclear cells or plasma (13, 53). These haplotypes appear to capture the majority of genetic variation in IL-18, due to the presence of strong linkage disequilibrium among the variants (3). Although these SNPs have been implicated in various disorders such as type I diabetes, asthma, hepatitis C virus, and rheumatoid arthritis (2, 15, 43), no studies have examined the relationships between the variants and malaria disease outcomes. As such, we examined the impact of IL-18 promoter polymorphisms (−137G→C and −607C→A) on susceptibility to severe falciparum malaria (Hb < 5.0 g/dl and any density parasitemia) and IL-18 transcriptional expression in children residing in a region where *P. falciparum* transmission is holoendemic in western Kenya.

(Portions of this work were presented at the 58th Annual Meeting of the American Society of Tropical Medicine and Hygiene held at Washington, DC, in 2009 [abstract 140].)
plates (Helena BioSciences, Sunderland, United Kingdom) according to the manufacturer's instructions. G6PD deficiency was determined by a fluorescent spot test using the manufacturer's methods (Trinity Biotech Plc., Bray, Ireland).

**Genetic analyses.** Blood spots were collected on FTA Classic cards (Whatman Inc., Clifton, NJ), air dried, and stored at room temperature until use. DNA was extracted using the Gentra system (Genta Systems, Inc., Minneapolis, MN) according to the manufacturer's recommendations. To obtain sufficient quantities for genetic analyses, genomic DNA was amplified using the GenomiPhi system (GE Healthcare, Piscataway, NJ). The IL-18—137G>C promoter polymorphism was genotyped by using the high-throughput TaqMan 5′ allelic discrimination Assay-By-Design method according to the manufacturer's instructions (assay identification number C_24108543_10; Applied Biosystems, Foster City, CA). Samples were genotyped for the −607C>A polymorphism by using the PCR-restriction fragment length polymorphism (RFLP) technique previously described (22).

**Extraction of RNA from WBC pellets.** Total RNA was extracted from frozen RNase-stored white blood cells (WBCs) by using the guanidine isothiocyanate (GITC)-isopropanol method as previously described (9). Briefly, WBCs were lysed for 5 min at room temperature in GITC solution containing 25 mM sodium acetate and 10% Sarkosyl. To completely dissociate the nucleoproteins, a layer of phenol was added, followed by chloroform-isomyl alcohol mix, vortexed thoroughly, and incubated for 30 min on ice. The aqueous phase was recovered after centrifugation and the extracted RNA precipitated overnight at −20°C in cold isopropanol. The precipitated RNA was thereafter pelleted by centrifugation, washed in 70% ethanol, and solubilized in RNase-free water. RNA was stored at −80°C until use.

**Reverse transcription-PCR and semiquantitative analysis of IL-18 gene expression.** To quantitatively estimate the extracted RNA, absorbance (A405/260) was measured using GeneQuant pro (GE Healthcare, Piscataway, NJ) for each sample, and concentrations were calculated based on optical densities. cDNA was synthesized using the TaqMan reverse transcription reagent kit (Applied Biosystems, Foster City, CA). Briefly, 1 μg of RNA was reverse transcribed in a 20-μl reaction volume mix containing, as final concentrations, 5 mM MgCl₂, 1 mM deoxyribonucleoside triphosphates (dNTPs), 1.5 mM (each) oligonucleotides with a sense sequence 5'-GTC TCC TTT GAG CTG TTT GC-3' and antisense 5'-AGA CTT CAG CAG GTG GCA GC-3' to generate a 301-bp fragment. To normalize the amount of cDNA loaded per reaction, an internal control, the cyclophilin A (CYC-A) housekeeping gene was amplified in a 25-μl reaction containing final concentrations of 0.3 mM each CYC-A sense oligonucleotide 5'-GTC TCC TTT GAG CTG TTT GC-3' and antisense oligonucleotide 5'-AAG CAG GAA CCC TTA TCA CC-3'. Amplification was carried out in a 96-well format GeneAmp PCR system 9700 (Applied Biosystems, Foster City, CA), in a reaction mixture containing final concentrations of 1.5 mM MgCl₂, 200 μM each dNTPs, 1.0 U GoTaq polymerase enzyme (Promega Corporation, Madison, WI), and 1× PCR buffer supplied by the manufacturer. Cycling conditions were as follows: initial denaturation at 94°C for 2 min, followed by 30 cycles each of denaturation at 94°C for 30 s, annealing at 67°C for 30 s, and 72°C for 45 s. A final extension of 72°C for 5 min was included, before cooling the products to 4°C. The resulting products were resolved on a 2% agarose gel stained with 0.5 μg/ml ethidium bromide (Sigma-Aldrich, St. Louis, MO) and visualized on an UV transilluminator (Spectroline Corporation, Westbury, NY). Digital gel photos were taken using a Canon PowerShot A640 camera (Canon Inc. Lake Success, NY) and band images were processed using ImageJ software (1). The integrated mean band intensities are reported as arbitrary units (AU) and were determined as ratios of the IL-18 gene band's mean intensity value divided by the corresponding housekeeping gene mean intensity to normalize the values. All HIV-1-positive and bacteremia-positive children were excluded from this analysis.

**Data analyses.** SPSS statistical software package version 15.0 (IBM SPSS Inc., Chicago, IL) was used for all statistical analyses. Demographic, clinical, and parasitological data between groups were compared using chi-square (χ²), Mann-Whitney U, and Student's t tests. Genotype, allele, and haplotypic frequencies of IL-18 were compared between non-SMA and SMA groups by using the χ² test. Pairwise comparisons of variables (genotypes and haplotypes) between non-SMA and SMA were conducted using the Mann-Whitney U test. IL-18 mRNA levels were normalized by expressing their mean absolute units (AU) as ratios of the CYC-A (housekeeping) gene. Parametric analyses between and within groups were performed using Student's t test (with Welch's correction) and analysis of variance (ANOVA) for bivariate and multivariate comparisons, respectively, both with 95% confidence intervals (CI). To assess the effect conferred by a particular genotype or haplotype (cross-sectionally), multivariate logistic regression analysis was used to calculate the odds ratio (OR) and 95% CI in a model controlling for the potential confounding effects of age, gender, G6PD deficiency, sickle cell trait, HIV-1 status, and presence of bacterial infections. The Hardy-Weinberg equilibrium (HWE) was tested using a χ² goodness-of-fit test. Linkage disequilibrium (LD) between polymorphisms was quantified using Multiallelic Interallelic Disequilibrium Analysis (MIDAS) software version 1.0 (12). Haplotypes were constructed using the HPlus software program (Fred Hutchinson Cancer Research Center, Seattle, WA) and their frequencies estimated based on a Bayesian algorithm. In addition, hierarchical logistic regression was used to investigate the association between haplotypes and longitudinal outcomes of repeated SMA episodes and mortality. Under this model, the covariates (i.e., age, gender, G6PD deficiency, sickle cell trait, HIV-1, and bacteremia status) were entered as independent confounding effects with haplotype contrast (carrier versus noncarrier) in predicting outcomes (SMA and mortality). All P values of <0.100 were further analyzed by using Cox regression/survival analysis, and differences in the distributions of hazard rate functions (i.e., the probabilities of experiencing the event) between carriers and noncarriers were examined using Mann-Whitney U tests. Statistical significance was defined by a value of <0.050 for all analyses.

**RESULTS**

**Characteristics of malaria-infected study participants upon enrollment.** Since one of the primary aims of the study was to determine the genetic variants that condition susceptibility to SMA in children with falciparum malaria, we first conducted cross-sectional analyses. As such, children with *P. falciparum* parasitemia (3 to 36 months; n = 523) were grouped according to the WHO definition of SMA, with non-SMA patients having Hb of ≥5.0 g/dl (n = 400) and SMA patients having Hb of <5.0 g/dl (n = 123) (48). A summary of demographic, clinical, and parasitological characteristics of the parasitemic study participants (upon enrollment) is shown in Table 1. The gender ratios in the clinical groups were comparable (P = 0.098). However, age differed across the groups, with the SMA group being significantly younger (P = 0.017). Consistent with the *a priori* stratification, Hb levels differed between the groups (P < 0.001). The admission temperature was, however, comparable between the groups (P = 0.364). Mean parasite densities (MPS/μl), geometric mean parasitemias (per μl), and the proportions of children with high-density parasitemia (HDP; MPS ≥ 10,000/μl) were not significantly different between the groups (P values of 0.340, 0.984, and 0.209, respectively). Similarly, the distributions of carriers of sickle cell traits (Hb AS, carriers [heterozygous] of the sickle cell trait) and glucose 6 phosphate (G6PD) deficiency were comparable between non-SMA and SMA groups (P = 0.110 and 0.210, respectively).

**Association between IL-18 gene expression and anemia in children with *P. falciparum* infections.** Prior to embarking on the genetic analyses, gene expression profiles of IL-18 were measured in non-SMA (n = 55) and SMA (n = 24) groups upon enrollment by use of semiquantitative mRNA analyses, normalized to the housekeeping gene cyclophilin A. Although there were higher IL-18 mRNA expression levels in children with SMA, the difference between the groups did not reach statistical significance (P = 0.068; Fig. 1a). An identical pattern was observed when children were categorized according to the definition using an Hb value of <6.0 g/dl as the cutoff criterion for SMA (P = 0.053; data not shown). Additional analysis of
TABLE 1. Demographic, clinical, and laboratory characteristics of the study participants

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Non-SMA patients</th>
<th>SMA patients</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of patients</td>
<td>400</td>
<td>123</td>
<td></td>
</tr>
<tr>
<td>No. (%) of:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Males</td>
<td>213 (53.9)</td>
<td>55 (44.7)</td>
<td>0.098</td>
</tr>
<tr>
<td>Females</td>
<td>187 (46.8)</td>
<td>68 (55.3)</td>
<td></td>
</tr>
<tr>
<td>Age (mo)</td>
<td>10.0 (10.0)</td>
<td>8.0 (7.0)</td>
<td>0.017</td>
</tr>
<tr>
<td>Hemoglobin (g/dl)</td>
<td>7.1 (2.8)</td>
<td>4.2 (1.1)</td>
<td>0.001</td>
</tr>
<tr>
<td>WBCs (×10³/µl)</td>
<td>11.2 (5.8)</td>
<td>15.2 (9.6)</td>
<td>0.001</td>
</tr>
<tr>
<td>Temperature (°C)</td>
<td>37.5 (2.0)</td>
<td>37.5 (2.0)</td>
<td>0.364</td>
</tr>
<tr>
<td>Parasite density (MPS/µl)</td>
<td>18,983 (39,222)</td>
<td>14,952 (44,241)</td>
<td>0.340</td>
</tr>
<tr>
<td>Geometric mean parasites/µl</td>
<td>13,849</td>
<td>10,667</td>
<td>0.984</td>
</tr>
</tbody>
</table>

a WBCs, white blood cells; MPS, malaria parasites; HDP, high-density parasitemia; G6PD deficiency, glucose-6-phosphate dehydrogenase deficiency.

The relationship between IL-18 mRNA expression and hemoglobin levels revealed that higher IL-18 transcripts were associated with lower hemoglobin concentrations (P = 0.293; P = 0.010) (Fig. 1b).

**Distribution of IL-18 promoter genotypes and alleles in children with acute malaria.** To investigate the association between IL-18 promoter variants (−137G>C and −607C>A) and cross-sectional susceptibility to SMA, genotype and allele frequencies were determined in non-SMA and SMA groups (Fig. 2a and b). Genotypic frequencies for the polymorphic variant at −137 were 0.78 (GG), 0.20 (GC), and 0.02 (CC) in the non-SMA group and 0.79 (GG), 0.19 (GC), and 0.02 (CC) in the SMA group, respectively (Fig. 2a). Both frequencies were consistent with HWE (non-SMA, χ² = 0.863 [P = 0.353]; SMA, χ² = 0.132 [P = 0.716]). Overall genotype distributions in the two groups were 0.78 (GG), 0.20 (GC), and 0.02 (CC) and were in equilibrium (χ² = 0.990; P = 0.320). The genotypic proportions between non-SMA and SMA groups were not significantly different (P = 0.934; Fig. 2a). Frequencies of the −137G and C alleles were 0.88 and 0.12 for non-SMA cases and 0.89 and 0.11 for the SMA group, respectively. The overall proportions were 0.88 and 0.12 for G and C alleles, respectively, and did not significantly differ between the groups (P = 0.853).

Genotypic distributions for the variants at −607 were 0.33 (CC), 0.50 (CA), and 0.17 (AA) in the non-SMA group and 0.42 (CC), 0.50 (CA), and 0.08 (AA) in the SMA group (Fig. 2b). Distributions in both groups were in equilibrium (non-SMA, χ² = 0.416 [P = 0.519]; SMA, χ² = 1.466 [P = 0.226]). Overall frequency distributions were 0.35 (CC), 0.50 (CA), and 0.15 (AA) and were consistent with HWE (χ² = 1.102; P = 0.314). The proportions of genotypes in the non-SMA and SMA groups were marginally different (P = 0.056; Fig. 2b). Distribution of alleles C and A of the −607 variant were 0.58 and 0.42 for the non-SMA group and 0.66 and 0.34 for SMA, respectively. Overall proportions were 0.60 and 0.40 for C and A alleles, respectively, and did not significantly differ between the non-SMA and SMA groups (P = 0.121).

**Distribution of IL-18 promoter haplotypes in children with acute malaria.** Construction of haplotypes for the two polymorphic loci yielded the following overall frequencies: 0.11 for −137G/−607A (CA), 0.01 for CC, 0.29 for GA, and 0.59 for GC. Distribution of haplotypes in children with SMA (n = 123) were 0.32 for CA, 0.17 for CC, 0.22 for GA, and 0.26 for GC (Fig. 2c). Comparison of SMA percentages among haplotypic carriers and noncarriers revealed that the group with the GC haplotype had a higher proportion of SMA patients (P = 0.034) and lower hemoglobin concentrations (P = 0.029) than the group with the non-GC haplotype (Fig. 2c). The proportions of chil-

![FIG. 1](https://example.com/figure1.png) Relationship between IL-18 mRNA expression and anemia. IL-18 gene expression was measured in peripheral blood leukocytes collected from children (n = 77) with malaria upon enrollment in the study (day 0). (a) Bivariate analysis of normalized IL-18 gene expression (arbitrary units [AU]) in the non-SMA (Hb ≥ 5.0 g/dl; n = 53) and SMA (Hb < 5.0 g/dl; n = 24) groups was performed using Student’s t test. (b) Association between normalized IL-18 mRNA levels (n = 77), expressed as AU, and hemoglobin levels (Hb; g/dl) at admission was determined by the Spearman rank correlation test.
children with SMA did not significantly differ for any of the other two haplotypes (non-CA versus CA, \( P = 0.826 \); non-CC versus CC, \( P = 0.691 \)) but marginally differed between non-GA and GA (\( P = 0.074 \)) haplotypes. Consistent with this distribution for the three haplotypes, Hb levels were comparable between haplotypic carriers and noncarriers (non-CA versus CA, \( P = 0.381 \); non-CC versus CC, \( P = 0.857 \); non-GA versus GA, \( P = 0.255 \)). Additional analyses demonstrated that the two loci were in linkage disequilibrium (LD; \( D' = 0.889 \)).

Relationship between IL-18 polymorphisms and SMA. To investigate the relationship between the IL-18 polymorphisms and susceptibility to SMA in children with acute disease, we performed multivariate logistic regression modeling, controlling for covariates. Analysis of the \(-137 \) variants showed no association between SMA and the homozygous mutant (CC, OR = 0.934; panel a) and \(-607 \) (\( P = 0.056 \); panel b). Analysis showed that there were more GC haplotypes in the SMA category than non-GC haplotypes (\( P = 0.034 \)). Additionally, analysis of Hb levels of non-GC and GC haplotypes revealed a significant difference (\( P = 0.029 \)). All the other haplotypes were comparable between carriers (haplotypes present) and noncarriers (haplotypes absent) (panel c).

Association between IL-18 haplotypes and SMA. Multivariate modeling of the haplotypes, controlling for covariates, showed that there was no cross-sectional association between susceptibility to SMA and carriage of the \(-137C/-607A \) (CA; OR = 0.966 [95% CI = 0.581 to 1.605], \( P = 0.983 \)) or CC (OR = 0.441 [95% CI = 0.048 to 4.040], \( P = 0.469 \)) haplotype. However, the GA haplotype showed a trend toward protection (\( P = 0.725 \) [95% CI = 0.478 to 1.099], \( P = 0.130 \)) against SMA (Fig. 3c). Furthermore, children with the GC haplotype demonstrated a risk of developing SMA 2-fold higher than that for children without the haplotype (OR = 2.050 [95% CI, 1.037 to 4.054], \( P = 0.039 \)). Consistent with these analyses, when <6.0 g/dl Hb was used to define SMA, there was no association between susceptibility to SMA and the CA (OR = 0.440 [95% CI = 0.212 to 0.856], \( P = 0.031 \)) (Fig. 3b). Heterozygosity (CA) at \(-607 \) showed a similar pattern and was associated with a 22% decrease in susceptibility to SMA (OR = 0.786 [95% CI = 0.741 to 1.280], \( P = 0.525 \)).
Levels of IL-18 transcripts in the genotypic groups. IL-18 transcript levels were quantified and compared across the genotypic groups (Fig. 4). Due to a lower prevalence of homozygous C individuals in the population, we were unable to obtain mRNA levels for this genotypic group. As such, we carried out a bivariate analysis to compare the mRNA levels in the two available genotypes (GG [n = 58] and GC [n = 19]). Results revealed that the transcriptional levels were comparable between the genotypes (P = 0.524; Student's t test) (Fig. 4a).

IL-18 mRNA levels across the −607 genotypes were marginally different (P = 0.081; ANOVA) (Fig. 4b). Additional bivariate analyses demonstrated comparable expression levels for the CA (n = 37, P = 0.751; Student’s t test) genotype relative to wild-type (CC, n = 31) genotype, as well as significantly different levels between AA (n = 9, P = 0.025; Student’s t test) and the CC genotype and also between the CA and AA (P = 0.047) groups.

Levels of IL-18 transcripts in the haplotypic groups. Stratification of IL-18 mRNA expression according to haplotypes showed that IL-18 transcripts were similar between carriers and noncarriers of the CA (n = 18; P = 0.466) (Fig. 4c) haplotypes. IL-18 levels could not be determined in the rare −137C/−607C (CC) haplotype, since no peripheral blood samples were available for this group. Carriers of the GA haplotype had marginally lower IL-18 transcripts (n = 30; P = 0.058) (Fig. 4d) than those with the non-GA haplotypes. IL-18 mRNA levels in carriers of the GC haplotype were observed to be significantly higher than those in the non-GC group (n = 68; P = 0.026) (Fig. 4e).

Association between haplotypes and longitudinal outcomes (SMA and mortality). After we determined the cross-sectional...
relationship between genotypes/haplotypes and susceptibility to SMA, hierarchical logistic regression was used to investigate the relationship between carriage of the different haplotypes and longitudinal outcomes (i.e., repeated episodes of SMA and mortality). Haplotypic distributions for the overall cohort (*n* = 719) were 0.11 (CA), 0.01 (CC), 0.29 (GA), and 0.59 (GC), consistent with those documented cross-sectionally. In addition, as with the cross-sectional analyses, the two loci were in linkage disequilibrium (*D* = 0.837) for the entire cohort. Longitudinal analyses failed to identify any significant relationships between repeated episodes of SMA and the CA (*P* = 0.530), CC (*P* = 0.788), GA (*P* = 0.543), and GC (*P* = 0.410) haplotypes. Over the 3-year follow-up period, there was an 8.2% mortality rate (59/719). Longitudinal modeling via hierarchical logistic regression revealed that there was a 33.3% mortality rate (4/12) in carriers of the CC haplotype and 7.8% (55/707) in noncarriers (*P* = 0.066). Consistent with these results, Cox regression modeling, controlling for the same confounding variables, revealed mean hazard rates (the probability of dying over time) of 37.2% and 6.5% for carriers and noncarriers of the CC haplotype and 7.8% (55/707) in noncarriers (β = −1.727, *P* = 0.066). Consistent with these results, Cox regression modeling, controlling for the same confounding variables, revealed mean hazard rates (the probability of dying over time) of 37.2% and 6.5% for carriers and noncarriers of the CC haplotype, respectively (*P* = 0.001). Thus, there was a 5.72 higher risk of all-cause mortality in carriers of the CC haplotypes. The longitudinal mortality did not differ between carriers and noncarriers of the CA (β = 0.499, *P* = 0.320), GA (β = −0.128, *P* = 0.710), and GC (β = −0.992, *P* = 0.184) haplotypes.

**DISCUSSION**

While uncovering genes that condition susceptibility to malaria is clearly important for an improved understanding of the disease, our laboratory has taken a different strategy. For example, since nearly all children in regions where transmission is holoendemic have repeated malarial infections, the question is not “What protects against acquisition of malaria?” but rather “What genes/gene pathways are associated with susceptibility to SMA?” with SMA being the single severe disease manifestation in regions of holoendemicity. Utilizing such an approach, focusing specifically on innate immune response genes in parasitized children, we have identified a number of genes that have significant relationships with both the risk of developing SMA and functional changes in their respective gene products (4, 37, 38). To extend these results, we investigated two functional promoter polymorphisms in IL-18 at positions −137 and −607 in children with and without SMA.

Prior to embarking on discerning the relationship between genotypes/haplotypes and susceptibility to SMA, we first determined if IL-18 gene expression differed between nonsevere and severe infections. Contrary to previous observations (8,
24), our results demonstrated that children with SMA had significantly higher levels of IL-18 transcripts. However, consistent with other studies (20, 29), we demonstrate that levels of IL-18 mRNA were inversely associated with Hb concentrations, suggesting that IL-18 may play an important role in conditioning anemia outcomes in children with falciparum malaria. Differences between our findings and previous ones (8, 24) are likely due to differences in study design and populations. For example, a previous study investigated responders and nonresponders to IL-18 among adult populations (8), while the current study evaluated IL-18 gene expression in children <36 months in age. In addition, severe malaria in previous studies was defined by mixed clinical phenotype (24), as opposed to our population in which SMA was the primary clinical outcome. It will be important to replicate the study in a different pediatric population experiencing SMA as the primary clinical outcome.

To determine if the relationship between IL-18 and anemia outcomes was conditioned by genotype, we first compared (cross-sectionally) the variant frequencies of the two loci independently. Although the SNPs at −137 were not different between the non-SMA and SMA groups, there was a marginal difference in frequency distribution for the −607 variants. In addition, the overall genotypic frequencies for the −137 and −607 SNPs in the non-SMA and SMA groups were consistent with HWE. Genotype frequencies for the −137 variants reported here are comparable to those documented in the HapMap database for the African ancestry population (GG, 0.696; GC, 0.304). Notably, the population investigated here possessed the homozygous CC (mutant) at 0.02, which was absent in the Yoruba population (sub-Saharan African descent) whose data are contained in HapMap (17). Single-locus minor allele frequencies (0.12 for −137C) closely resemble those for the Yoruban population (i.e., 0.15). Similarly, the −607 variant frequencies in the Kenyan children examined here are comparable to those for the Yoruban population (0.43 for CC, 0.44 for CA, and 0.12 for AA). The minor allele frequency (−607A) in the Kenyan children (0.40) showed a trend similar to that reported for the Yoruba population (0.34). Taken together, these results suggest that IL-18 promoter alleles and genotypes have been maintained in ethnic groups of African descent.

Functional assays have previously demonstrated that the −137C and −607A alleles are associated with decreased production of IL-18 (3, 13). Variant −137 is located within multiple nuclear binding sites, which include the human histone H4 gene-specific transcription factor 1 (H4TF1), hepatocyte nuclear factor-3β (forkhead box A2), and the Th2-specific transcription factor GATA binding protein 3 (607). In 2001, Giedraitis et al. (13) observed that transversion from G to C at position −137 changes the H4TF1 nuclear binding site into that for an unknown factor located within the granulocyte-macrophage colony-stimulating factor (GM-CSF) promoter, potentially reducing production of IL-18. Furthermore, the C-to-A change at the second loci we investigated (−607) mediates transcriptional activation in response to cAMP by disrupting the binding site, which may also result in lower IL-18 production.

Since some studies have shown an association between elevated IL-18 levels and severe P. falciparum malaria (20, 29), we hypothesized that single-locus substitutions resulting in decreased IL-18 production would potentially protect against SMA. However, multivariate modeling revealed that single-locus variants at position −137 were not significantly associated with susceptibility to SMA. Consistent with this finding, IL-18 mRNA expression did not appear to be conditioned by −137 variants. Previous studies showing that −137 variants may differentially regulate IL-18 production in liver hepatocyte cells (6, 16), presence of IL-18 in liver cells (40), and the role of IL-18 in T cell-mediated liver injury (45) suggest that −137 variants could potentially be important in the preerythrocytic (liver) stage of malaria. However, the study design employed here, examining the erythrocytic stage of disease, cannot address this hypothesis.

Cross-sectional investigation of variation at −607 demonstrated that the −607AA (mutant) genotype was significantly associated with protection against SMA and showed a tendency toward protection when SMA was defined as <6.0 g/dl Hb. In addition, although the differences in IL-18 mRNA levels between the genotypic groups did not significantly differ across the groups, carriers of the AA genotype had the lowest IL-18 levels. Post hoc analyses showed that IL-18 transcripts were significantly lower in the AA group than for both CC and CA carriers. Taken together, these findings support the notion that reduced levels of IL-18, conditioned, at least in part, by variation at −607, are associated with protection against SMA.

Since haplotypes often reveal how combinations of different functional polymorphic alleles interact to amplify, or moderate, their individual effects (38, 51), we constructed haplotypes for the two loci. Consistent with previous reports (39, 42), there was linkage disequilibrium between the −137 and −607 loci in our study population. Stratification of children according to haplotypes showed that the group with the −137G/−607C (GC) haplotype had a significantly higher proportion of SMA patients and significantly lower Hb concentrations. Additional analyses using multivariate logistic regression, controlling for confounders, revealed that GC carriers had twice the risk of developing SMA compared to children without the haplotype. Further investigation using the modified definition of SMA (Hb < 6.0 g/dl) showed a similar trend toward increased risk.

Investigation of IL-18 mRNA expression in the haplotypic groups revealed that children with the GC haplotype also had significantly higher levels of IL-18 transcripts. These results are consistent with previous studies demonstrating that the GC and GA haplotypes had higher transcriptional activity than the CA and CC haplotypes (13, 53). Given the significantly elevated transcript levels of IL-18 in the SMA group, coupled with results showing that the GC haplotype is associated with increased risk of SMA and significantly higher IL-18 levels, we propose that elevated IL-18 augments SMA pathogenesis. This hypothesis is supported by previous investigations showing that elevated IL-18 levels are associated with severe malaria outcomes (20, 29).

Investigation of the relationship between haplotypes and susceptibility to SMA over the 3-year follow-up did not show any significant association between haplotypes and repeated episodes of SMA. These results suggest that although IL-18 promoter haplotypes are important in conditioning susceptibility to SMA in children once they acquire falciparum malaria, they do not appear to influence either the acquisition of re-
peated malaria infections (data not presented) or future SMA episodes. However, there was a 4-fold-higher all-cause mortality rate among carriers of the −137C→−607C (CC) haplotype according to hierarchical logistic regression modeling. Additional investigation of the relationship between carriage of the CC haplotype and mortality by using Cox regression modeling, controlling for the same confounding variables, demonstrated that the probability of mortality over time was 5.72-fold higher in carriers of the CC haplotype. The high rate of mortality in this group is consistent with the exceedingly low CC haplotypic frequency, which represented 1% of the total haplotypic constructs. Although it remains to be determined, the low frequency of the CC haplotype in the children examined here, and the complete absence of the CC haplotype in the adult Yoruban population, may indicate a selection bias due to its association with mortality.

In conclusion, we report for the first time that IL-18 promoter haplotypes (IL-18 −137G→−607C→A) are important in conditioning the development of SMA in children residing in an area where P. falciparum transmission is holoendemic. Based on data presented here, carriers of the GC haplotype appear to have a genetic predisposition to develop SMA once they acquire a P. falciparum infection, at least in part, through overproduction of IL-18. In addition, although the haplotypes examined here do not appear to influence susceptibility to either malaria or SMA longitudinally, carriage of the CC haplotype appears to be an important genetic factor that impacts on childhood mortality. Future longitudinal investigations aimed at determining the exact cause of death in carriers of the CC haplotype will be required to determine how inheritance of this haplotype impacts on childhood mortality.

ACKNOWLEDGMENTS

We offer our sincere gratitude and appreciation to all parents, guardians, and children from the Siaya District Community for their participation in this study. We also thank the staff at University of New Mexico/KEMRI laboratories, including Anne A. Ong’ondo, Chrispine W. Gogen, L. A. Ochicha, Nicholas K. Gomma, Rodmey B. Mingare, Salome A. Yala, Moses Epungure, Fabian Oduor, and Joseph Oduor, as well as the Siaya District Hospital management team, for their support during the study. These data presented are published with the permission and approval of the Director, Kenya Medical Research Institute.

The study was funded from National Institutes of Health (NIH) grant R01AI15305 (D.J.P.) and Fogarty International Center (FIC) training grant 1 D43TW05884 (D.J.P.). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

We declare that no competing interests exist.

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Editor: J. H. Adams


