

Published in final edited form as:

Hum Genet. 2012 August ; 131(8): 1375–1391. doi:10.1007/s00439-012-1175-1.

Reduced interferon (IFN)- α conditioned by *IFNA2* (–173) and *IFNA8* (–884) haplotypes is associated with enhanced susceptibility to severe malarial anemia and longitudinal all-cause mortality

Prakasha Kempaiah¹, Samuel B. Anyona², Evans Raballah², Gregory C. Davenport¹, Tom Were^{2,3}, James B. Hittner⁴, John M. Ong'echa^{1,2}, and Douglas J. Perkins^{1,2,*}

¹Center for Global Health, Department of Internal Medicine, University of New Mexico Health Sciences Center, Albuquerque, NM, USA

²University of New Mexico/KEMRI Laboratories of Parasitic and Viral Diseases, Kisumu, Kenya

³Department of Pathology, Kenyatta University, Nairobi, Kenya

⁴Department of Psychology, College of Charleston, Charleston, SC, USA

Abstract

Severe malarial anemia (SMA) is a leading cause of pediatric morbidity and mortality in holoendemic *Plasmodium falciparum* transmission areas. Although dysregulation in cytokine production is an important etiology of SMA, the role of IFN- α in SMA has not been reported. As such, we investigated the relationship between IFN- α promoter polymorphisms [i.e., *IFNA2* (A-173T) and *IFNA8* (T-884A)], SMA, and functional changes in IFN- α production in children (n=663; <36 mos.) residing in a holoendemic *P. falciparum* transmission region of Kenya. Children with SMA had lower circulating IFN- α than malaria-infected children without severe anemia ($P=0.025$). Multivariate logistic regression analyses revealed that heterozygosity at –884 (TA) was associated with an increased risk of SMA [OR, 2.80 (95% CI, 1.22–6.43); $P=0.015$] and reduced IFN- α relative to wild-type (TT; $P=0.038$). Additional analyses demonstrated that carriage of the –173T/–884A (TA) haplotype was associated with increased susceptibility to SMA [OR, 3.98 (95% CI, 1.17–13.52); $P=0.026$] and lower IFN- α ($P=0.031$). Follow-up of these children for 36 mos. revealed that carriers of TA haplotype had greater all-cause mortality than non-carriers ($P<0.001$). Generation of reporter constructs showed that the *IFNA8* wild-type –884TT exhibited higher levels of luciferase expression than the variant alleles ($P<0.001$). Analyses of malaria-associated inflammatory mediators demonstrated that carriers of TA haplotype had altered production of IL-1 β , MIG, and IL-13 compared to non-carriers ($P<0.050$). Thus, variation at *IFNA2*–173 and *IFNA8*–884 conditions reduced IFN- α production, and increased susceptibility to SMA and mortality.

*Address correspondence to: Douglas J. Perkins, Ph.D., ¹Center for Global Health, Department of Internal Medicine, University of New Mexico Health Sciences Center, MSC10-5550, Albuquerque, NM 87131-0001, USA. Phone: 505-272-6867; fax: 505-272-8441, dperkins@salud.unm.edu.

COMPETING INTEREST STATEMENT

The authors have declared that no competing interests exist.

The study was approved by the ethical and scientific review committees at the University of Pittsburgh and University of New Mexico, and the Kenya Medical Research Institute. A portion of this work was presented previously at the 58th ASTMH annual meeting held at Washington, D.C., abstract # 742.

Keywords

Interferon; promoter polymorphism; SMA; inflammatory mediators

INTRODUCTION

Malaria is an infectious disease caused by parasites of the genus *Plasmodium*. Of the five primary human forms of malaria causing over 1.24 million deaths per year, *Plasmodium falciparum*, accounts for greater than 1 million deaths, with more than 3.3 billion people at risk of infection (Murray et al. 2012). Among the various clinical sequelae in *P. falciparum* infections, the most prevalent severe disease manifestation is severe malarial anemia (SMA) that occurs primarily in children <5 years of age, resulting in ~22% of the malaria-associated childhood deaths in Africa (Obonyo et al. 1998). SMA results from a combination of hematological factors including, direct and indirect destruction of parasitized red blood cells (pRBC), increased clearance of uninfected erythrocytes, and suppression of the erythropoietic response (Keller et al. 2009; Kurtzhals et al. 1997; Were et al. 2006; Wickramasinghe and Abdalla 2000). In holoendemic *P. falciparum* transmission regions, such as our study site in western Kenya, we have found that inefficient erythropoiesis is a primary etiology of SMA; a process that is largely influenced by an imbalance in inflammatory mediators (Ong'echa et al. 2011; Othoro et al. 1999; Ouma et al. 2010; Ouma et al. 2006; Perkins et al. 2011; Were et al. 2006).

Along these lines, the current study focused on the role of interferon (IFN)- α in conditioning SMA. Although the role of IFN- α , particularly in human malaria, is largely unexplored, we previously showed that reduced circulating IFN- α levels were associated with severe falciparum malaria in Gabonese and Kenyan children (Luty et al. 2000; Ong'echa et al. 2011). In addition, studies in murine models of malaria demonstrated that treatment with recombinant huIFN- α resulted in reduced blood parasite levels and mortality from cerebral malaria, but had no effect on anemia outcomes (Vigario et al. 2001; Vigario et al. 2007). IFN- α is a pleiotropic cytokine in the type 1 interferon family that has been extensively used for decades as an immunotherapeutic agent in malignant, angiogenic, allergic, inflammatory, viral, parasitic, and autoimmune diseases (Beilharz et al. 1997; Bogdan 2000; Ferbas et al. 1994; Muller et al. 1994; Vigario et al. 2001; Vilcek 2006). IFN- α is also an important regulator of cellular growth and differentiation that bridges innate and adaptive immune responses (Biron 1999). IFN- α is primarily produced and secreted by fibroblasts, macrophages, plasmacytoid monocytes, T cells, dendritic cells (DCs), and natural killer (NK) cells (Francis et al. 1996; Havell et al. 1978).

There are (at least) 14 different IFN- α intronless genes encoding for 13 identical mature proteins clustered on human chromosome 9p22 (Samuel 2001). However, due to post-translational modifications, there are more than 22 IFN- α subtypes differing by one or two amino acids (Bekisz et al. 2004; Song et al. 2006). Several *in vitro* studies showed that these subtypes vary considerably in their ability to produce IFN- α in response to viruses and other stimuli (Izaguirre et al. 2003; Seto et al. 1995).

Previous investigations demonstrated that polymorphic variability in the promoter region of *IFNA2* was associated with reduced transcription and susceptibility to hepatitis B and C viruses, but had no association with malaria disease severity (Song et al. 2006; Tena-Tomas et al. 2008). Among the IFN- α subtypes, *IFNA8* is one of the highest producers of IFN- α and possesses potent antiviral activity (Foster et al. 1996; Garcia et al. 2007; Izaguirre et al. 2003; Seto et al. 1995). However, *IFNA8* has not been investigated in the context of malaria.

Based on the allelic distribution reported for IFN- α polymorphic variability in reference African ethnic populations (NCBI_refSNP), we focused our investigations on *IFNA2* (A-173T) and *IFNA8* (T-884A) since these particular variants have minor allelic frequency (MAF) distributions that can generate comprehensive coverage in the population. The current study determined the association between individual promoter variants, their haplotypic constructs, and susceptibility to both SMA and mortality during acute malaria and throughout the development of naturally acquired malarial immunity in children (n=663) residing in a holoendemic *P. falciparum* transmission region of western Kenya. Functionality of the genetic variation was determined by examining the relationship between genotypes/haplotypes and circulating IFN- α , and through the generation of luciferase reporter constructs. In addition, we investigated the association between IFN- α genotypes/haplotypes and inflammatory mediators known to be dysregulated in malarial anemia using a 25-plex inflammatory array (Perkins et al. 2011).

MATERIALS AND METHODS

Study subjects

The study was conducted at Siaya District Hospital (SDH) in Siaya District, western Kenya, a holoendemic *P. falciparum* transmission area (Beier et al. 1994). In this region, SMA is the primary clinical manifestation of severe malaria in children under the age of five years, with cerebral malaria occurring only in rare cases (Bloland et al. 1999; McElroy et al. 2000; Ong'echa et al. 2006). A complete description of the clinical, demographic, and hematologic characteristics of the cohort is presented in our previous publication (Ong'echa et al. 2006). Children (n=663, age 3–36 mos.) were recruited during their first hospital visit for the treatment of malaria between 2003 and 2008. Although the global distribution of malaria has undergone a decrease, the rates of malaria-related morbidity and mortality have actually increased in Siaya during the time frame of the current study (Hamel et al. 2011). Children with non-falciparum malaria, cerebral malaria, prior transfusions, or previous hospitalization (for any reason) were excluded from the study. Since our previous studies demonstrated that HIV-1 and bacteremia are common in this area, (Otieno et al. 2006; Were et al. 2011), all children were tested for these pathogens (see procedures listed below). Once disease status was determined, the children were given appropriate treatment and supportive therapy according to Ministry of Health, Kenya guidelines. Pre- and post-test HIV counseling was provided for the parents/guardians of all study participants. Written informed consent in the language of choice (i.e., English, Kiswahili, or Dholuo) was obtained from the parents/guardians of participating children. The study was approved by the ethical and scientific review committees at the Kenya Medical Research Institute and the Institutional Review Board at the University of New Mexico.

Children with falciparum malaria were classified according to World Health Organization (WHO) definitions of malarial anemia: non-SMA (Hb \geq 5.0 g/dL, with any density parasitemia) and SMA (Hb $<$ 5.0 g/dL, with any density parasitemia) (WHO 2000). In addition, based on a previous study examining $>$ 14,000 longitudinal Hb measurements according to age, gender, and geographical context in children less than 48 months of age in western Kenya (McElroy et al. 1999), parasitemic children were stratified into two primary clinical groups: non-SMA (Hb \geq 6.0 g/dL, with any density parasitemia) and SMA (Hb $<$ 6.0 g/dL, with any density parasitemia).

Sample collection laboratory measures

Venous blood samples ($<$ 3.0 mL) were collected into EDTA-containing Vacutainer[®] tubes prior to administration of anti-malarials and/or any other treatment interventions. Asexual malaria parasites (trophozoites) were counted against 300 leukocytes in peripheral blood

smears stained with Giemsa reagent and parasite density was estimated as follows: parasite density/ μL =white blood cell (WBC) count/ μL \times trophozoites/300. Complete hematological parameters were determined with a Beckman Coulter[®] A^C.T diff²[™] (Beckman-Coulter Corporation, Fullerton, CA). Sickle-cell trait (HbAS), glucose-6-phosphate dehydrogenase (G6PD) deficiency, HIV-1 status, and bacteremia were determined according to our published methods (Ong'echa et al. 2006; Otieno et al. 2006; Ouma et al. 2010).

DNA extraction and genotyping

Genomic DNA was extracted from buccal swabs using the Buccal Amp[™] DNA extraction kit (Epicentre Biotechnologies, Madison, WI) and then amplified using GenomiPhi[™] (GE Healthcare Life Sciences, Amersham, UK). *IFNA2* A-173T and *IFNA8* T-884A SNPs were genotyped using the Taqman[®] 5'-allelic discrimination Assay-By-Design method according to the manufacturer's instructions (Assay ID's: C_33349900_10 and C_31289660_10, respectively) on the StepOnePlus[™] PCR system (Applied Biosystems, Foster City, CA).

Cell lines and culture

The human monocyte-macrophage cell line (U937) and human fibrosarcoma cell line (HT-1080) were obtained from American Type Culture Collection (ATCC, Manassas, VA). The monocyte-macrophage (U937) and fibroblast (HT-1080)-based cell lines were used to examine promoter variants, since IFN- α is primarily produced and secreted by monocyte/macrophages, fibroblasts, and other cells (Havell et al. 1978). U937 cells were maintained in RPMI-1640 medium (ATCC, Manassas, VA), supplemented with 10% fetal bovine serum (FBS, Gibco-Invitrogen, Carlsbad, CA), penicillin (100 U/mL), and streptomycin (0.1 mg/mL) (Sigma-Aldrich, St Louis, MO), while the HT-1080 cell line was maintained in Dulbecco's modified Eagle medium (DMEM) (Mediatech, Manassas, VA), supplemented with 10% heat-inactivated FBS, and penicillin-streptomycin. Cells were cultured at 37°C in a humidified atmosphere containing 5% CO₂.

Construction of reporter plasmids

For all reporter constructs, the fragment of the *IFNA8* promoter region (as determined by the genome analyzer and search engine at <http://www.genomatix.de>) from -961 to +39 (1kb) was synthesized by PCR using the genomic DNA template from individuals carrying the different variants at position -884 (T/A). The fragment was amplified using the forward primer - containing a *Sac* I (underlined) site (5'-TTTGAGCTCCAGAGTCATACCATGTGATG-3') and reverse primer- containing a *Nhe* I (underlined) site (5'-TTTGCTAGCATAGGGAACATATTTGTTTC-3'), and Phusion[®] high-fidelity DNA polymerase kit (Finnzymes, Woburn, MA). PCR conditions were: one cycle for 1 min at 98°C, for an initial denaturation step; followed by 32 cycles at 98°C for 10 s; annealing for 30 s at 58°C; and extension for 30 s at 72°C; followed by a final extension step for 10 min at 72°C. PCR products were then purified over Microcon[®] centrifugal filter devices (Millipore, Bedford, MA) and digested with restriction enzymes. The gel-extracted product was cloned directly into *Sac* I and *Nhe* I sites of a promoter-less luciferase reporter gene vector, pGL3-Basic (Promega, Madison, WI). Positive clones (carrying the promoter region) were cut with the same enzymes to confirm presence of the insert. To verify the -884 promoter variants and ensure no additional variation was present, sequencing was performed using the BigDye[®] Terminator Cycle Sequencing Kit in a Model 377 Sequencing System (Applied Biosystems, Foster City, CA).

Transient transfection and luciferase assay

Promoter constructs were prepared using an endotoxin-free plasmid DNA isolation kit (Qiagen, Valencia, CA). For transient transfection of reporter vectors, U937 cells (2×10^5

cells) were freshly added to 12-well plates, while HT-1080 cells (1.5×10^5 cells) were grown 24 h prior to transfection in 12-well plates. The pGL3-control vector, pGL3-basic and test reporter constructs (1 μ g each), were separately mixed with Opti-MEM[®] I reduced serum medium and Lipofectamine[™] 2000 reagent (Invitrogen, Carlsbad, CA), and transfection was performed according to manufacturer's instructions. Cells were incubated at 37°C for 5 h in a 5% CO₂ atmosphere. After transfection, cells were further incubated with (stimulated) or without (basal) 50 ng/mL phorbol 12-myristate 13-acetate (PMA) and 1.5 μ M ionomycin (Sigma-Aldrich, St. Louis, MO) in 1 mL of fresh medium for different time periods. At selected intervals, cells were harvested, washed once with phosphate-buffered saline (PBS, Invitrogen, Carlsbad, CA), and lysed using the passive lysis buffer (Promega, San Luis Obispo, CA) following the supplier's protocol. Twenty microliters of the lysates were used to measure luciferase activity in a Lumat LB9501 luminometer (EG & G Berthold, Oak Ridge, TN) using the dual luciferase[®] reporter assay system kit (Promega, San Luis Obispo, CA). Relative luciferase activity was calculated by dividing the raw data of the test reporter's gene expression to pGL3-basic data, and the transfection efficiency was verified using the pGL3-control plasmid. All assays were conducted in duplicate in three independent experiments and the mean value from each experiment was selected for data analyses.

Nitrate and nitrite quantitation

Systemic nitric oxide (NO) production [nitrite plus nitrate levels (NO_x)] was quantified in urine samples according to our previous methods using *Aspergillus* nitrate reductase coupled with the Griess reaction (Perkins et al. 1999). Creatinine (Cr) levels were measured using a commercially available colorimetric assay (Creatinine Assay Kit, Cayman Chem., Ann Arbor, MI). Urinary NO_x concentrations were expressed as a ratio of NO_x/Cr (μ M/ μ M) to account for potential differences in renal function.

Multiplex assay

Plasma samples obtained from venous blood were stored at -80°C until use. Samples were then thawed and clarified by centrifugation (14,000 rpm for 10 min) before assaying. Inflammatory mediators levels were determined by the Cytokine 25-plex Antibody Bead Kit, Human (BioSource[™] International, Camarillo, CA) according to the manufacturer's instructions. Plates were read on a Luminex[®] 100[™] system (Luminex[®] Corporation, Austin, TX) and analyzed using the Bio-plex manager software (version IS.2.3; Bio-Rad Laboratories, Hercules, CA). Analyte detection limits were: 3pg/mL (IL-5, IL-6, IL-8/CXCL8); 4pg/mL [monokine induced by IFN- γ (MIG/CXCL9)]; 5pg/mL (IL-1 α , IL-3, IL-4, IL-10, eotaxin-1/CCL11, IFN- γ , IP-10/CXCL10); 6pg/mL (IL-2); 10pg/mL (IL-7, IL-13, IL-15, IL-17, MIP-1 α /CCL3, MIP-1 β /CCL4, monocyte chemoattractant protein [MCP-1/CCL2], TNF- α); and 15pg/mL (IL-1 β , IL-12p40/p70, granulocyte-macrophage colony-stimulating factor [GM-CSF], IFN- α , RANTES/CCL5).

Longitudinal follow-up

Upon enrollment of the children into the study (n=663, Day 0), parents/guardians were asked to return with their child every 3 mos. throughout a 36 mos. follow-up period. If the parent/guardian had not returned to hospital by 1:00 pm on the day of the quarterly follow-up visit, our study staff visited the child's residence to check on their health status, including mortality. Since we determined the exact location of each child's residence with our GIS/GPS surveillance system, we could readily locate each child. In addition, since children experience multiple episodes of malaria, and other pediatric infectious diseases, in this region, parents/guardians were asked to return to hospital during their child's febrile episode(s). All laboratory tests required for proper clinical management of the patients were performed at each acute and quarterly visit, including complete hematological indices,

malaria parasitemia measures, and evaluation of bacteremia (if clinically indicated). In addition, all-cause mortality data were collected throughout the three year follow-up. Mortality data, clinical and laboratory measures for multiple episodes of malaria were used to evaluate the association between IFN- α haplotypes and longitudinal outcomes of SMA and mortality. Although most children within this region die at home, visits by our study team confirmed the date of mortality.

Statistical analyses

Statistical analyses were performed using SPSS (version 15.0 for Windows, SPSS Inc.). Kruskal-Wallis test was used to compare medians across the groups, while pairwise comparisons of medians were performed using Mann-Whitney U test. Chi-square (χ^2) analyses and Fisher's exact test were used to examine differences in gender, parasite density, and genotype and haplotype proportions between non-SMA and SMA groups. IFN- α promoter haplotypes were constructed using HPlus software (version 4.0 for windows 7) and the distribution frequencies of SNPs and haplotypes were determined using the Haploview (version 4.2). Multivariate logistic regression analyses, controlling for co-variables, including age, gender, G6PD deficiency, HIV-1 status, bacteremia, and sickle-cell trait (Aidoo et al. 2002), was used to examine the association between IFN- α variation and SMA outcomes. Deviation from Hardy-Weinberg Equilibrium (HWE) was determined using web-based software (Court 2008). Co-efficient (D') of pairwise linkage disequilibrium (LD) between SNPs was calculated using MIDAS software (version 1.0) (Gaunt TR 2006). Analyses of luciferase reporter assay data were performed using the GraphPad Prism 5[®] (GraphPad Software Inc. La Jolla, CA) software. Hierarchical logistic regression was used to investigate the association between IFN- α haplotypes on longitudinal outcomes of SMA episodes and mortality. For the analyses using 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 vs. non-carrier) in predicting outcomes (SMA and mortality). All values of $P < 0.100$ were further analyzed using Cox regression/survival analysis and the mean hazard rates for haplotype groups between carriers and non-carriers were examined using non-parametric Mann-Whitney U and Kolmogorov-Smirnov Z tests. The hierarchical logistic regression analyses were conducted by entering two sequential blocks of predictor variables: Block 1 consisted of Age, Gender, G6PD, Sickle cell status, bacteremia, and HIV status (and the intercept term for the model, b_0). Block 2 consisted of haplotype status (carrier vs. non-carriers). For both of our longitudinal outcome variables (i.e., mortality status and SMA episodes), the equation for the logit function is the same. That is, the probability of the event occurring, $P(Y)$, is a function of the intercept and the seven predictors listed. The specific logistic regression equation (logit function) is presented below:

$$P(Y) = \frac{1}{1 + e^{-(b_0 + b_1 \text{Age} + b_2 \text{Gender} + b_3 \text{G6PD} + b_4 \text{SickleAA vs. AS} + b_5 \text{Bacteremia} + b_6 \text{HIV} + b_1 \text{Haplotype} + \epsilon)}}$$

Statistical significance for all analyses was set at $P < 0.050$.

RESULTS

Demographic and clinical characteristics of the study participants

Children ($n=508$) from day 0 with *P. falciparum* infections were stratified into two groups based on Hb concentrations: non-SMA (Hb ≥ 5.0 g/dL, $n=384$) and SMA (Hb < 5.0 g/dL, $n=124$) in the cross-sectional analyses. The demographic and clinical characteristics of the study participants are shown in Table 1. SMA was characterized by younger age ($P=0.047$),

increased axillary temperature ($^{\circ}\text{C}$, $P=0.024$), and a higher WBC count ($10^3/\mu\text{L}$) ($P<0.001$). As expected based on the *a priori* grouping, Hb (g/dL), hematocrit (%), and RBC counts ($10^{12}/\text{L}$) were lower in the SMA group ($P<0.001$, $P<0.001$ and $P<0.001$, respectively). The mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC) were significantly different in SMA group ($P<0.001$, $P=0.018$, and $P=0.033$, respectively). Reticulocyte indices such as absolute reticulocyte number (ARN), reticulocyte production index (RPI) and $\text{RPI}<2$ (an indicator of inefficient erythroid production) were significantly lower in SMA group ($P=0.005$, $P=0.032$ and $P=0.008$, respectively). Additional hematological indices such as lymphocytes, monocytes, granulocytes and platelets counts were also significantly different between the groups ($P<0.001$, $P<0.001$, $P=0.003$, and $P=0.028$, respectively). None of the other parameters differed significantly between the two groups. Although there was not enough sample available to perform a comprehensive differential diagnosis of anemia, the non-significant difference in the RDW, and the significantly elevated MCV and MCHC in the SMA group, suggest that iron deficiencies may not be a primary cause of severe anemia in this cohort. Moreover, our previous report in this cohort of children showing comparable levels of acanthocytes in the non-SMA and SMA groups supports this notion (Novelli et al. 2010). Consistent with our previous results illustrating significantly higher numbers of nucleated RBCs in children with SMA (Novelli et al. 2010), and our finding that inefficient erythropoiesis is a primary cause of SMA in this region (Awandare et al. 2011; Were et al. 2006), the SMA group presented here had a significantly lower RPI and greater percentage of children with an $\text{RPI}<2.0$, suggesting that severe anemia is due, at least in part, to bone marrow abnormalities.

Circulating IFN- α in the non-SMA and SMA groups

Prior to investigating the impact of variation in the *IFNA2* and *IFNA8* loci on SMA, circulating IFN- α was examined in the non-SMA and SMA groups. Since co-infection(s) could potentially affect the levels of IFN- α , all co-infected children were excluded from the analyses. As shown in Figure 1, IFN- α levels [median, range (Q1-Q3)] were lower in children with SMA [12.50 (5.0–69.75), $n=66$] compared to those with non-SMA [39.0 (5.0–90.0), $n=177$, $P=0.025$]. Based on the significant difference in IFN- α levels, we then proceeded with genotyping the *IFNA2* (A-173T) and *IFNA8* (T-884A) loci followed by construction of their respective haplotypes.

Distribution of IFN- α genotypes and haplotypes

Genotypic distributions of the individual *IFNA2* (A-173T) and *IFNA8* (T-884A) loci and their haplotypic constructs in the non-SMA ($n=384$) and SMA ($n=124$) groups, as well as the overall cohort ($n=508$) are shown in Table 2. The overall allele [major/minor (p/q)] frequencies for the polymorphisms were: A-173T (0.77/0.23); and T-884A (0.89/0.11), respectively. The frequencies for A-173T were not in HWE for both the non-SMA ($\chi^2=5.09$, $P=0.023$) and SMA ($\chi^2=6.80$, $P=0.009$) groups, and overall cohort ($\chi^2=11.433$, $P<0.001$). However, the distribution of genotypes for *IFNA2* between the non-SMA and SMA groups did not differ ($P=0.814$). The frequencies of the T-884A locus were not in HWE in the non-SMA ($\chi^2=23.70$, $P<0.001$) and SMA ($\chi^2=31.14$, $P<0.001$) groups, and the overall cohort ($\chi^2=45.51$, $P<0.001$). Although non-significant, there was an enhanced frequency of the A allele in the non-SMA group relative to children with SMA ($P=0.080$).

After examining the two SNPs independently, haplotypes were then constructed. The haplotypic distributions were: AT (0.69), TT (0.20), AA (0.08), and TA (0.03). Comparison of the four haplotypes between the non-SMA and SMA groups revealed a higher distribution of individuals with the TA haplotype in the non-SMA group ($P=0.072$). The AT, TT, and

AA haplotypes did not significantly differ between non-SMA and SMA groups ($P=0.385$, $P=0.311$, and $P=0.252$, respectively).

Examination of linkage disequilibrium (LD)

Additional analyses showed that there was no evidence of LD between the SNPs (A-173T/T-884A): ($D'=0.099$; $r^2=0.004$).

Association between IFN- α genotypes/haplotypes and SMA

To determine the effect of individual promoter variants and haplotypes on susceptibility to SMA, multivariate logistic regression was performed controlling for the confounding effects of age, gender, G6PD deficiency, sickle-cell trait, HIV-1 status, and bacteremia (Aidoo et al. 2002; Okwara et al. 2004; Ong'echa et al. 2006; Otieno et al. 2006; Ouma et al. 2006; Were et al. 2011). Relative to the AA genotype at the *IFNA2*-173 locus, the AT (OR; 1.24, 95% CI, 0.74–2.07; $P=0.406$) and TT (OR; 1.27, 95% CI, 0.53–3.05; $P=0.587$) genotypes were not associated with susceptibility to SMA [using either WHO (Hb <5.0 g/dL) or modified definition (Hb <6.0 g/dL)] (Figure 2). Analysis of the *IFNA8*-884 locus revealed that relative to TT individuals, carriage of the TA genotype was associated with increased susceptibility to SMA according to the WHO and modified definitions of disease (Hb <5.0 g/dL, OR; 2.80, 95% CI, 1.22–6.43; $P=0.015$, Figure 2 and Hb <6.0 g/dL, OR; 2.19, 95% CI, 1.23–3.90; $P=0.008$, data not shown). However, homozygosity for the A allele was not associated with susceptibility to SMA (OR; 0.91, 95% CI, 0.32–2.62; $P=0.874$, Figure 2).

Multivariate logistic regression modeling of the haplotypes, controlling for identical covariates, revealed that carriage of the TA (-173T/-884A) haplotype was associated with significantly higher risk of developing SMA (Hb <5.0 g/dL, OR; 3.98, 95% CI, 1.17–13.52; $P=0.026$, Figure 2, and Hb <6.0 g/dL, OR; 2.68, 95% CI, 1.24–5.82; $P=0.012$, data not shown). There was no significant association between the AT (Hb <5.0 g/dL, OR; 1.0, 95% CI, 0.5–2.0; $P=0.939$), AA (Hb <5.0 g/dL, OR; 1.48, 95% CI, 0.66–3.3; $P=0.331$), and TT (Hb <5.0 g/dL, OR; 0.98, 95% CI, 0.6–1.61; $P=0.953$) haplotypes and susceptibility to SMA using either WHO definition (Figure 2) or modified disease definition (data not shown). Thus, there was an increased risk of developing SMA in individuals with the TA genotype at the *IFNA8*-884 locus (OR=2.80) that was further enhanced in individuals carrying a haplotype that contained the T and A alleles at *IFNA2*-173 and *IFNA8*-884, respectively (OR=3.98).

Relationship between IFNA genotypes/haplotypes and IFN- α

To examine potential functionality associated with the genotypes/haplotypes, levels of IFN- α were compared across the groups after stratifying according to *IFNA2*-173 and *IFNA8*-884 genotypes, and their haplotypes. These analyses revealed that variation at the *IFNA2*-173 locus was not associated with circulating IFN- α levels ($P=0.198$, Figure 3). Analysis of the *IFNA8*-884 locus demonstrated that IFN- α was significantly different across the groups ($P=0.045$, Figure 3). Individuals with TT genotype had the highest IFN- α levels compared to TA group [21.0 (5.0–74.50) vs. 7.0 (5.0–45.50), $P=0.038$]. IFN- α levels were lowest in individuals carrying the AA genotype, but did not differ significantly from those with the TT genotype [5.0 (1.0–48.50), $P=0.860$] (Figure 3).

Further analyses examining the haplotypes showed that individuals with the TA (-173T/-884A) haplotype had significantly lower circulating IFN- α levels than the non-TA group [7.0 (5.0–31.50) vs. 18.0 (5.0–74.50), $P=0.031$, Figure 3]. None of the other haplotypes were associated with significantly different IFN- α levels. Taken together, these results illustrate that the *IFNA8*-884 TA genotype and the TA haplotype which conditioned increased risk of developing SMA were associated with significantly lower levels of circulating IFN- α .

Analysis of variant specific constructs

Functional variation within promoter regions can dramatically affect transcriptional activity. *In silico* transcription factor binding site (TFBS) analysis (<http://www.cbil.upenn.edu/cgi-bin/tess/tess>) indicated that a T to A transition at the –884 loci of *IFNA8* can abrogate the binding site for HOXA5 (Homeobox protein Hox-A5, IMD matrix 100184) factor, while no known TFBS were identified at –173 locus. Based on this information and the fact that variation at the –884 locus, but not the –173 locus was associated with susceptibility to SMA, only the –884 locus was investigated for transcriptional activity using variant specific constructs in a luciferase-based expression system. In addition, generating constructs for haplotypes composed of alleles from both the –173 and –884 loci was not feasible since they are located in two (separate) haploblocks (analyzed using SNPbrowser 3.0™ program, NCBI build 36). The pGL3-TT, pGL3-TA, and pGL3-AA constructs for the –884 locus were created using genomic DNA from the study participants with the respective genotypes as a template for PCR amplification. As shown in Figure 4, there was significantly higher basal activity for the wild-type (TT) construct versus either the TA or AA constructs in both cell lines at 24 and 48 hrs ($P < 0.001$ for all comparisons). Although the wild-type promoter construct (pGL3-TT) showed increased gene expression following stimulation with PMA and ionomycin in both cell lines at 24 and 48 hrs, particularly in the HT-1080 cell line, induction of gene expression was completely lacking in the presence of the polymorphic alleles (Figure 4). No significant differences between the TA and AA constructs were observed at any of the time points under basal or stimulated conditions for both cell lines ($P > 0.50$ for all comparisons, Figure 4). These results demonstrate that the wild type allele is associated with both higher baseline and stimulated gene expression than the polymorphic alleles, supporting the *in vivo* observations in children with falciparum malaria.

Potential mechanisms of anemia: inhibition of erythropoiesis

The mechanism(s) by which reduced IFN- α could promote enhanced anemia in human malaria are unclear. Our previous results in the cohort examined here demonstrated that one of the primary mechanisms responsible for SMA was suppression of erythropoiesis (Awandare et al. 2011; Were et al. 2006). In addition, previous studies in a murine model of malaria illustrated that administration of recombinant IFN- α reduced blood-stage disease through its ability to decrease the number of reticulocytes induced following infection (Vigario et al. 2001). To determine the association between genotypes/haplotypes and the erythropoietic response, we performed additional multivariate modeling for the specific genetic variants that were associated with significantly higher risk of developing SMA and lower circulating IFN- α (i.e., the *IFNA8* –884 TA genotype and the TA haplotype). For these analyses, the dependent variable in the multivariate logistic regression modeling (controlling for identical co-variates) was a reticulocyte production index (RPI) < 2.0 based on the fact that this particular measure is indicative of inefficient erythropoiesis (Were et al. 2006). Relative to the TT genotype at the *IFNA8* –884 locus, carriage of the ‘susceptible’ TA (OR; 1.07, 95% CI, 0.429–2.69; $P = 0.878$) or AA (OR; 1.843, 95% CI, 0.399–8.519; $P = 0.434$) genotypes were not associated with an inefficient erythropoietic response. In addition, carriage of the ‘susceptible’ TA haplotype, relative to non-carriers, was not associated with reduced erythropoiesis (OR; 1.081, 95% CI, 0.307–3.806; $P = 0.903$). Thus, there does not appear to be a direct association between reduced erythropoiesis and carriage of either the TA genotype or TA haplotype, suggesting that other mechanisms are responsible for the enhanced risk of SMA in these children.

Potential mechanisms of anemia: soluble mediators of inflammation

Our previous investigations, focused on mechanisms of anemia in children with malaria, have shown that soluble mediators of inflammation play an important role in conditioning anemia outcomes (Keller et al. 2009; Ong'echa et al. 2011; Ouma et al. 2010; Were et al.

2006). In addition, we recently showed that several inflammatory mediators (i.e., nitric oxide and TNF- α), that are associated with anemia outcomes in children with malaria, also directly inhibit erythropoiesis in a novel *in vitro* model developed in our laboratory (Awandare et al. 2011). To further investigate potential mechanisms by which variation in *IFNA* subtypes could promote SMA, we determined the association between the 'susceptible' genetic variants (i.e., the *IFNA8*-884 TA genotype and TA haplotype) and a panel of circulating inflammatory mediators (n=26). Stratification of the *IFNA8*-884 genotypes revealed that carriage of the 'susceptible' TA genotype was associated with significantly lower levels of NO_x/Cr [median, range (Q1-Q3) 0.014 (0.011–0.025) vs. 0.026 (0.017–0.040), $P=0.008$] and MIG [123.7 (81.58–201.5) vs. 187.0 (98.67–319.9), $P=0.006$] compared to the TT genotype (Figure 5A). However, although the carriage of the AA genotype was associated with lower NO_x/Cr [0.038 (0.004–0.067)] and MIG [95.19 (66.69–589.4)], these results were not statistically significant vs. the TT genotype ($P=0.235$ and $P=0.632$, respectively, Figure 5A). None of the other inflammatory mediators examined were significantly different across the genotypic groups (data not presented).

When inflammatory mediators were stratified according to carriage of the TA haplotype, the 'susceptible' carriers of the TA haplotype had significantly lower circulating MIG levels [136.7 (78.70–161.2), vs. 180.5 (92.14–310.1), $P=0.031$, Figure 5B] relative to non-carriers of the haplotype. Although NO_x/Cr was lower in carriers of the TA haplotype, the results were not significant [0.022 (0.012–0.028) vs. 0.026 (0.016–0.040), $P=0.580$, Figure 5A] relative to non-carriers. Additional analyses revealed that carriers of the TA haplotype had significantly higher levels of IL-1 β [334.4 (195.6–692.4) vs. 170.0 (72.32–345.0), $P=0.020$] and IL-13 [41.80 (26.30–75.12) vs. 23.40 (9.08–45.30), $P=0.020$], and marginally higher levels of RANTES [22,912 (4,118–14,0554) vs. 11,313 (4,437–42,358), $P=0.066$] (Figure 5C) relative to non-carriers of the TA haplotype. None of the other inflammatory mediators differed significantly between the TA and non-TA haplotypes (data not presented). Results presented here show that the carriage of the 'susceptible' *IFNA8*-884TA genotype and TA (-173T/-884A) haplotype are associated with altered levels of inflammatory mediators that we have previously shown are associated with susceptibility to malarial anemia (Keller et al. 2004; Ong'echa et al. 2011; Were et al. 2006).

Association between IFN- α haplotypes and longitudinal outcomes (mortality and SMA)

After examining the cross-sectional relationship between 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., mortality and SMA). Haplotypic distributions for the overall cohort (n=663) were: 0.64 (AT), 0.08 (AA), 0.22 (TT), and 0.06 (TA), consistent with those documented cross-sectionally. In addition, as with the cross-sectional data analyses, the two loci were not in LD ($|D'|=0.096$, $r^2=0.004$) for the cohort. Longitudinal modeling via hierarchical logistic regression revealed that there was a 14.5% mortality rate (8/55) in carriers of the TA haplotype and 8.1% (49/608) in non-carriers ($\beta=-0.921$, $P=0.060$). Consistent with these results, Cox regression modeling, controlling for the same confounding variables as those in the cross-sectional modeling, revealed mean hazard rates (the probability of dying over time) of 0.1467 and 0.0587 for carriers and non-carriers of the TA haplotype, respectively ($P<0.001$) (Figure 6). Thus, there was a 2.5-fold higher risk of all-cause mortality in carriers of the TA haplotypes. However, the longitudinal mortality did not differ between carriers and non-carriers of the AT ($\beta=-0.004$, $P=0.995$), AA ($\beta=-513$, $P=0.339$) and TT ($\beta=0.283$, $P=0.488$) haplotypes.

Examination of the association between haplotypes and repeated episodes of SMA over 36 mos. with hierarchical logistic regression modeling failed to find any significant relationship between SMA outcomes and carriage of AT ($\beta=0.131$, $P=0.642$), AA ($\beta=-0.095$, $P=0.732$), TT ($\beta=0.100$, $P=0.605$), and TA ($\beta=-0.399$, $P=0.195$) haplotypes.

DISCUSSION

IFN- α has long been described as a cytokine with a wide variety of biological functions including antiviral and immunoregulatory functions (Beilharz et al. 1997; Bogdan 2000; Ferbas et al. 1994; Muller et al. 1994). Studies using *in vitro* and *in vivo* models have also shown that IFN- α both directly, and indirectly, regulates the activity of a number of other cytokines and chemokines (Biron 2001; Brinkmann et al. 1993; Mori et al. 1998; Taylor and Grossberg 1998). A role for IFN- α in regulating the immune response during human malarial infections was first described in our previous study showing that suppression of IFN- α in Gabonese children was associated with enhanced severity of malaria, with severe disease characterized by a mixed clinical sequelae of anemia and/or hyperparasitemia (Luty et al. 2000). Subsequent investigations in murine models of malaria demonstrated that administration of recombinant IFN- α treatment resulted in lower peripheral parasite loads and reduced mortality from cerebral malaria (Vigario et al. 2001; Vigario et al. 2007). Results presented here support and extend previous human and animal studies by showing that reduced circulating IFN- α levels are associated with SMA and conditioned by specific genetic variants in the promoter region of *IFNA2* and *IFNA8*.

Several *in vitro* and *in vivo* studies have shown that promoter polymorphisms in IFN- α are associated with susceptibility to viral and parasitic infections, and altered transcriptional rates (Song et al. 2006; Tena-Tomas et al. 2008). To explore the impact of naturally occurring variation in the IFN- α promoter on malaria disease outcomes, we investigated two IFN- α subtypes, *IFNA2* (A-173T) and *IFNA8* (T-884A). Selection of these variants was based on the abundant MAF distribution in African populations that was available from information at (CHIP_bioinformatics; NCBI_refSNP). The high MAF distribution in the Kenyan cohort examined here (i.e., Luo ethnic group) are similar to the other Kenyan ethnic groups such as Luhya in Webuye (LWK), Maasai in Kinyawa (MKK) and Yoruban population [i.e., YRI, Yoruba people of Ibadan (0.22 for -173 locus and 0.11 for -884 locus)]. Interestingly, we identified no variation for these particular *IFN* subtypes in other ancestral groups (e.g., Asian, European, etc.) (Kwiatkowski 2005; NCBI_refSNP). Although the reason for this phenomenon is difficult to define, it is consistent with the notion that selective pressure exerted on the human genome by malaria, and other infectious diseases, in African populations has created extensive variation in immune response genes that is not witnessed in other ancestral groups (Kwiatkowski 2005; NCBI_refSNP). Therefore, these results further reinforce the notion that the genetic diversity has been enhanced in immune response genes in ethnic groups from sub-Saharan African. Analyses of the allelic distribution of the *IFNA2*-173 and *IFNA8*-884 promoter variants revealed a significant departure from HWE for both loci in the overall cohort, and in the non-SMA and SMA groups. However, these two loci were not in linkage disequilibrium.

To determine the effects of individual genotypic variants on susceptibility to SMA, we performed multivariate logistic regression analyses, controlling for the confounding effects of age, gender, G6PD deficiency, sickle-cell trait, HIV-1 status, and bacteremia (Aidoo et al. 2002; Okwara et al. 2004; Ong'echa et al. 2006; Otieno et al. 2006; Ouma et al. 2008; Ouma et al. 2006; Were et al. 2011). In addition, hepatitis B and C could (potentially) influence our results. However, based on implementation of the HBV vaccine in November of 2001 in Kenya (Ndiritu et al. 2006), and the fact that all of the children in the cohort received this vaccination as part of their routine childhood immunizations, HBV is not a likely confounding variable. Although studies from this geographic region show that the HCV infection rate in adults is 0.2–0.9% (Muasya et al. 2008), no data are available regarding HCV for pediatric populations in the region. To control for the possibility of HCV infection, we used clinical and laboratory data to exclude all children from the analyses who presented with jaundice, hepatomegaly (particularly, inflammation at the right lower rib margin), and

“coffee-colored” urine. As such, the phenotypically well-defined cohort studied here offers a unique advantage for investigating the genetic basis of susceptibility to severe malaria as a single disease entity without the confounding effects of co-infection. Results from the multivariate modeling revealed that heterozygous individuals (TA) at *IFNA8*-884 locus had a significantly higher risk of developing SMA. Construction of haplotypes and additional modeling, controlling for identical co-variables, demonstrated that carriage of the -173T/-884A (TA) haplotype increased susceptibility to SMA to an even greater extent than that observed for the *IFNA8*-884 TA genotype. These findings support our previous investigations showing that polymorphic variation in the promoter regions of innate immune response genes are important for conditioning susceptibility to SMA (Anyona et al. 2011; Keller CC 2009; Ouma et al. 2008; Ouma et al. 2010; Ouma et al. 2006).

To further determine the potential functional consequences of variation in the *IFNA2*-173 and *IFNA8*-884 loci, data were stratified according to genotypes/haplotypes and circulating levels of IFN- α were examined. These analyses showed that the TA genotype at the *IFNA8*-884 locus and the TA (-173T/-884A) haplotype, both of which conditioned significantly higher risk of developing SMA, were also associated with significantly lower IFN- α levels. Thus, our previous findings in Gabonese children showing progressive reduction in circulating IFN- α with increasing malaria severity (Luty et al. 2000), along with results presented here, suggest that reduced IFN- α production, at least in part, influences the development of severe malaria.

Based on the genotypic results for the *IFNA8*-884 locus, in conjunction with lower levels of circulating IFN- α , and *in silico* analysis suggesting potential abrogation of the HOXA5 binding site, variant specific constructs were created for this locus. This series of experiments demonstrated that the wild-type promoter construct (pGL3-TT) had high levels of basal expression that was further up-regulated in response to stimulation. In contrast, both the heterozygous (pGL3-TA) and homozygous (pGL3-AA) constructs had low levels of basal activity that was largely unresponsive to stimulation with PMA and ionomycin. These results, along with the relatively similar risks associated with susceptibility to SMA for the *IFNA8* TA genotype (OR=2.80) and the -173T/-884A (TA) haplotype (OR=3.98), and their comparable reductions in circulating IFN- α , suggests that it is actually variation at *IFNA8*-884 that is driving the genetic-based relationship and consequent changes in IFN- α . These results are consistent with previous studies showing that the *IFNA8* subtype is one of the most important type 1 interferon subtypes for regulating potent IFN- α production (Foster et al. 1996; Garcia et al. 2007; Izaguirre et al. 2003; Seto et al. 1995).

The exact mechanism by which IFN- α regulates anemia outcomes is largely unknown. Potential mechanisms may include IFN- α acting directly on the erythropoietic process or alternatively, through indirect effects in which IFN- α acts on other inflammatory mediators within the bone marrow milieu. A previous study in a murine model of malaria, using a parasitic strain that preferentially invade reticulocytes, showed that IFN- α treatment reduces blood-stage disease through its ability to reduce the overall number of reticulocytes (Vigario et al. 2001). This, along with our previous investigation demonstrating that pediatric SMA in western Kenya is characterized by suppression of erythropoiesis (Awandare et al. 2011; Keller CC 2009; Ong'echa JM 2011; Ouma et al. 2010; Were et al. 2006) prompted us to determine the impact of the ‘susceptible’ genotype (*IFNA8* TA genotype) and haplotype (-173T/-884A) on the erythropoietic response (i.e., a ‘direct’ mechanism). To our surprise, neither the ‘susceptible’ genotype nor haplotype were associated with reduced erythropoiesis (i.e., an RPI<2.0), suggesting an alternative explanation for the association between *IFNA* genetic variation and susceptibility to SMA.

We then investigated the hypothesis that *IFNA* genetic variation may condition SMA by indirectly influencing the inflammatory milieu. As such, a panel of inflammatory mediators was examined in the 'susceptible' groups. This included measurement of circulating cytokines and chemokines, and urinary NO_x/Cr. Consistent with our previous results showing that IFN- α is a potent inducer of human NOS2-derived NO production (Kun et al. 2001), the low IFN- α -producing -884TA genotype was also associated with significantly lower NO_x/Cr. Although NO_x/Cr was also reduced in the low IFN- α -producing TA haplotype, the reduction was not significant (possibly due to reduced sample size, n=6), therefore, making it difficult to draw any definitive conclusions about the potential role of NO in promoting anemia in this group of children.

Examination of the inflammatory milieu in carriers versus non-carriers of the TA haplotype yielded interesting findings. We have previously shown that functional variation in the promoter of IL-1 β that produces higher levels of IL-1 β production promotes an increased risk of SMA (Ouma et al. 2008). Consistent with these findings, carriers of the 'susceptible' TA haplotype had significantly higher circulating IL-1 β levels. In addition, individuals with the TA haplotype also had significantly higher levels of IL-13. Although a role for IL-13 in the context of SMA, and human malaria in general, has not been previously reported, recent results from our laboratory aimed at identifying biomarkers of malarial anemia found that elevated levels of IL-13 are a highly significant predictor of SMA (Ong'echa et al. 2011). Since IL-13 is typically associated with alternative activation of macrophages and immunity against extracellular parasites (Martinez et al. 2009), it remains to be determined if IL-13 plays a causal role in the pathogenesis of malaria or if it is simply a marker of type 2 immunity being released to counter-act the over-expression of type 1 cytokines characteristic of SMA. However, based on the fact that IL-13 directly inhibits CFU-E *in vitro* (Sakamoto et al. 1995; Xi et al. 1995), examination of IL-13 and its relationship to IFN- α production requires further investigation. The other significant finding that emerged from these investigations was the reduced circulating levels of MIG in the TA haplotype group. Levels of MIG were also significantly lower in the carriers of the TA genotype versus the TT and AA groups. As with IL-13, a role for MIG in promoting anemia in children with malaria has not been previously described and warrants further investigation.

Examination of the impact of IFN- α on the development of mortality and SMA throughout a 36 mos. longitudinal follow-up revealed that children with the TA haplotype had a 2.5-fold higher all-cause mortality rate than non-carriers. Association of this group with a high rate of mortality is consistent with the low frequency distribution of the haplotype (6% of the total haplotypes) which may indicate a selection bias towards low representation in the cohort. Since most of the study participants died at home, it was not possible to determine the exact cause of death. Thus, it is difficult to infer how the low IFN- α producing TA haplotype influenced all-cause mortality. Although definitively determining how the low IFN- α producing TA haplotype influences mortality will certainly require additional investigations, it is possible that individuals who produce low levels of IFN- α may be susceptible to a number of infectious diseases that are common to the region. This hypothesis is consistent with the fact that IFN- α plays an important central role in protection against a number of infectious diseases (Beilharz et al. 1997; Bogdan 2000; Garcia et al. 2007; Izaguirre et al. 2003; Luty et al. 2000; Ong'echa et al. 2011; Vigario et al. 2001; Vilcek 2006). Although SMA is a leading cause of mortality in the study region (Anyona et al. 2011; Brabin et al. 2001; Were et al. 2011), none of the haplotypes examined were significantly associated with SMA throughout the follow-up period. This may be largely related to the fact that the children died at home and, as such, SMA was not diagnosed, but could have been captured indirectly in the analyses examining all-cause mortality. In addition, it is possible that some of the haplotypes do, indeed, influence longitudinal outcomes, but were not identified in the hierarchical logistic regression and Cox regression

analyses since the block 2 haplotypic effects had decreased statistical power based on small and/or unbalanced haplotypic group sample sizes.

While the exact mechanism(s) through which IFN- α influenced anemia outcomes in the current cohort remains unclear, the association between the 'susceptible' genetic groups and altered levels of inflammatory mediators known to impact on anemia suggests that the biologic actions of IFN- α may occur through altering the inflammatory environment (an indirect mechanism). Future studies should aim to delineate the precise role of IFN- α and identify the downstream inflammatory mediators affected by altered IFN- α production in children with malaria.

In summary, results in the present study demonstrate that reduced IFN- α , conditioned by genetic variation, particularly at the *IFNA8* locus, promote enhanced anemia in Kenyan children with falciparum malaria. These data, along with our previous investigation in Gabonese children with malaria (Luty et al. 2000), and studies in animal models of malaria (Vigario et al. 2001; Vigario et al. 2007), all point to IFN- α playing a protective role in malarial disease. Based on these collective studies, and the abundant and long-standing clinical use of IFN- α to treat a number of inflammatory and infectious diseases, it raises the question as to whether IFN- α therapy may also have a role in treating malaria.

Acknowledgments

This work was supported by National Institutes of Health Research Grants-R01AI51305 and D43TW05884 (DJP). The authors gratefully acknowledge the assistance of the Siaya District Hospital team and the University of New Mexico/KEMRI staff for all the clinical support. We are very grateful to all the parents, guardians and, children that participated in the study. The authors sincerely thank the Director of Kenya Medical Research Institute for approving the data to be published in the manuscript.

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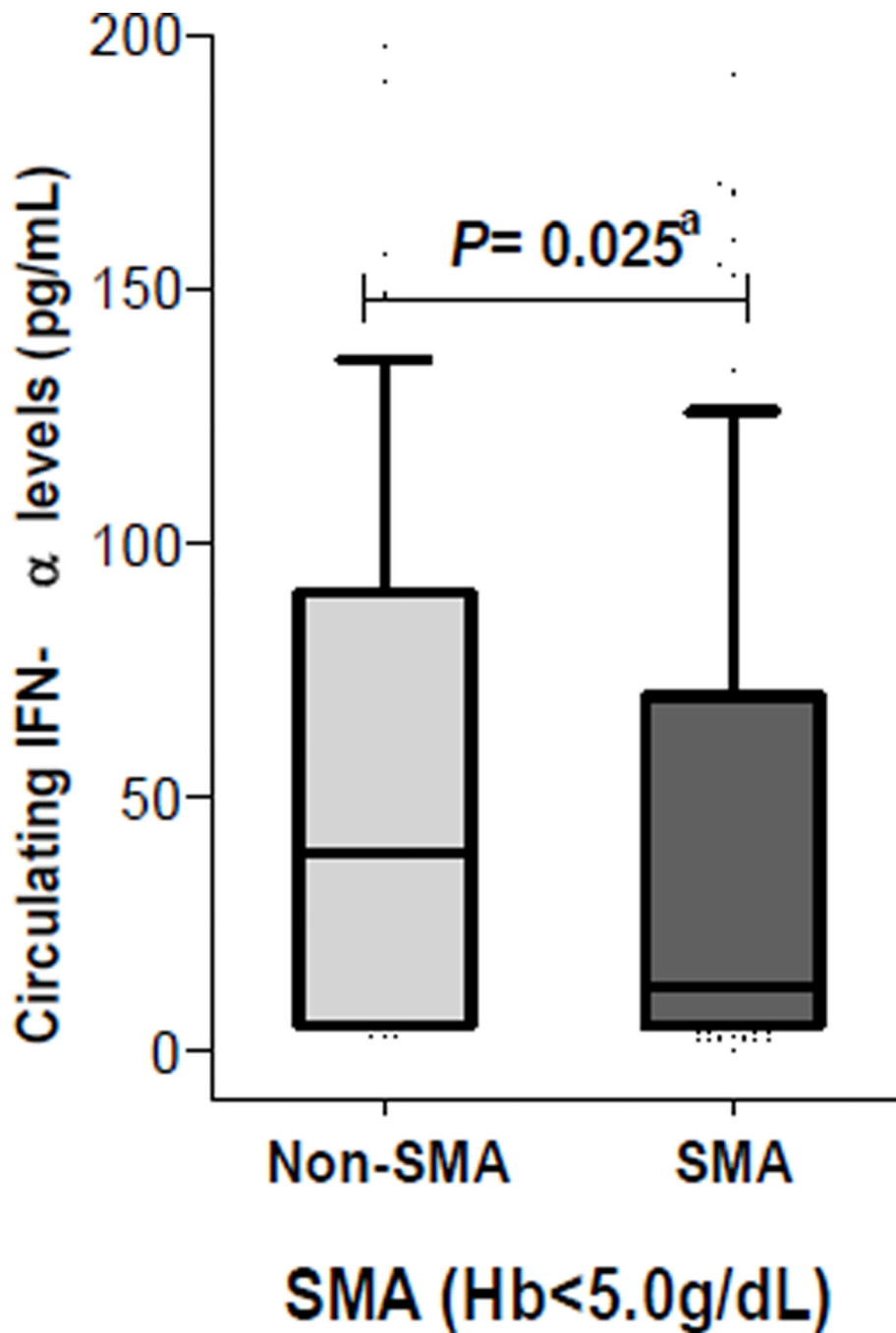


Figure 1. Determination of circulating IFN- α levels in parasitemic children

Concentrations of circulating IFN- α (pg/mL) in children aged <3.0 years with *P. falciparum* malaria was measured using the 'Human Cytokine Twenty-Five-Plex Antibody Bead Kit' as described in the Methods. Children (n=243) were categorized according to the WHO definition of SMA based on Hb concentrations (i.e., Hb<5.0 g/dL, with any density parasitemia). Results are presented for children with non-SMA (i.e., Hb \geq 5.0 g/dL, n=177) and SMA (i.e., Hb<5.0 g/dL, n=66). Box-plots depict the data where the box represents the interquartile range, the line through the box is the median, and whiskers illustrate the 10th and 90th percentiles. Significantly reduced circulating IFN- α levels were found in the SMA group compared to the non-SMA group ($P=0.025$, ^aMann-Whitney U test).

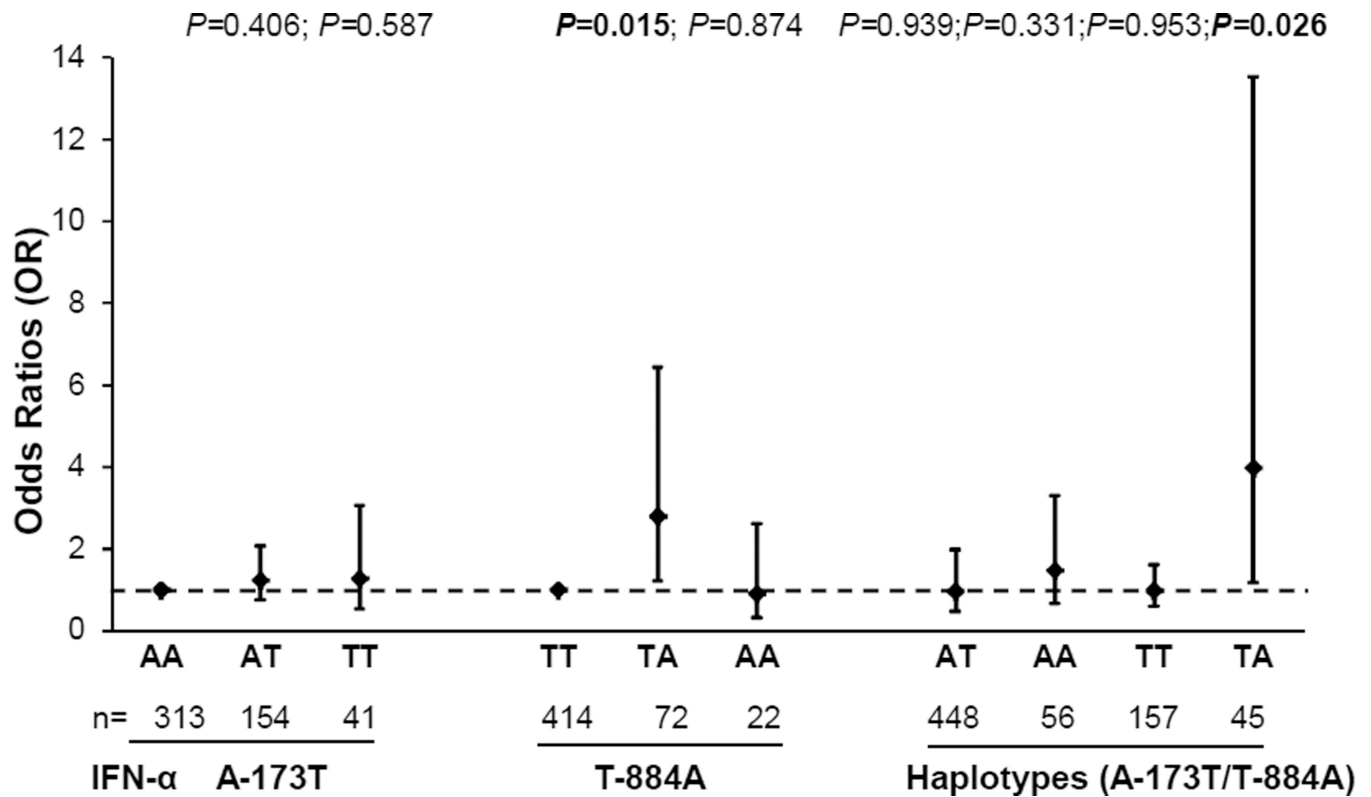


Figure 2. Effect of IFN- α genotypes and haplotypes on SMA

Parasitemic children (n=508) were categorized based on clinical categories of SMA (i.e., Hb<5.0 g/dL) and non-SMA (i.e., Hb \geq 5.0 g/dL). Data presented as Odds Ratios (OR) and 95% CI were determined using multivariate logistic regression analyses controlling for confounding factors [age, gender, HIV-1 status, G6PD deficiency, bacteremia, and HbAS]. In the multivariate analysis of genotypic data, the wild-type was used as reference category for individual SNPs. To determine the impact of each haplotype on disease phenotype, individuals without the haplotype were used as the reference group in the multivariate analyses. As shown in the figure, the presence of TA genotype at *IFNA8*-884 locus increases susceptibility to SMA and presence of the TA (-173T/-884A) haplotype was associated with an increased risk of developing SMA.

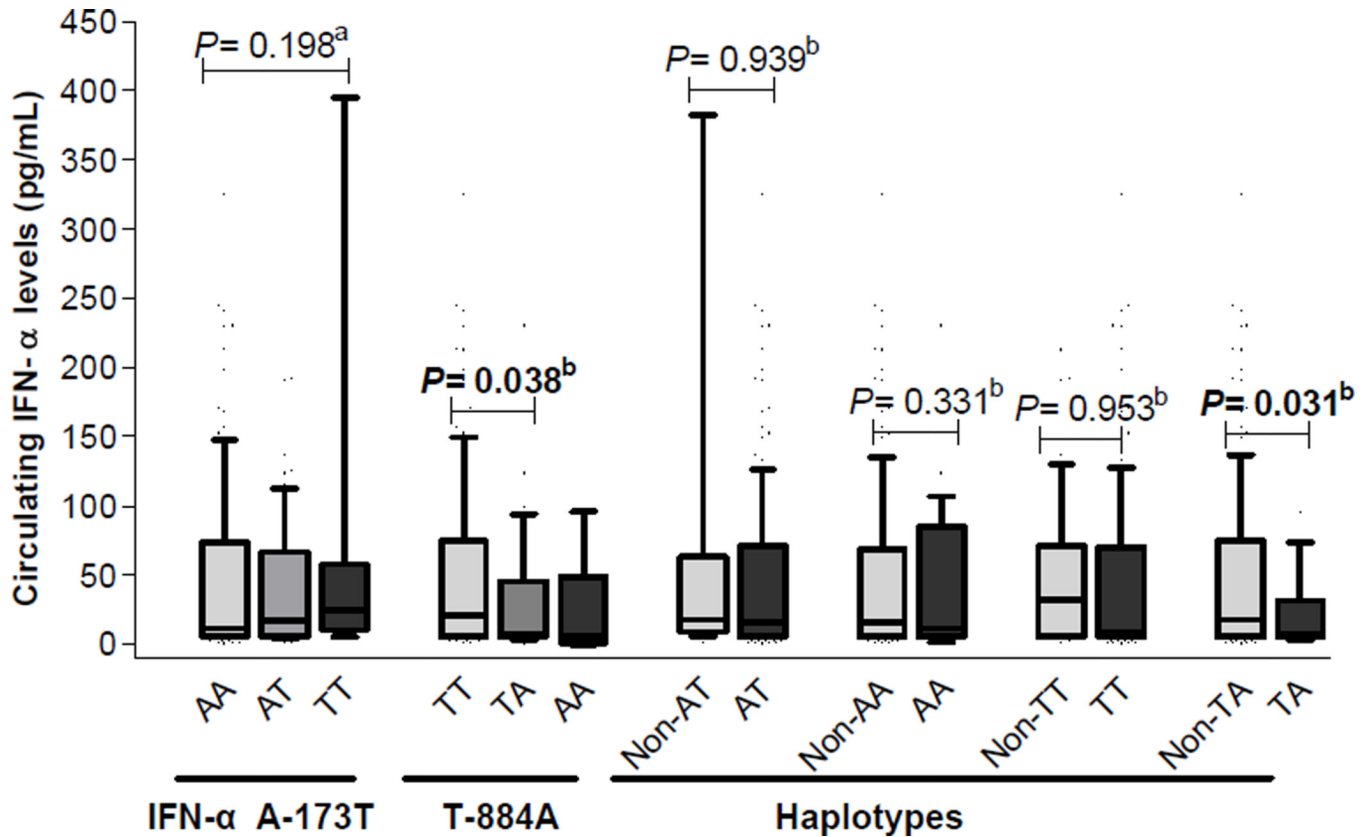


Figure 3. Association of circulating IFN- α levels with genotypic and haplotypic variants
Circulating IFN- α concentration (pg/mL) in parasitized children (n=243) aged <3.0 years was measured using the 'Human Cytokine Twenty-Five-Plex Antibody Bead Kit'. Results are presented for each IFN- α genotypic category; *IFNA2*, A-173T (AA, n=140; AT, n=84; TT, n=19) and *IFNA8*, T-884A (TT, n=194; TA, n=41; AA, n=8). In addition, results are also presented for carriers vs. non-carriers of the *IFNA2* and *IFNA8* haplotypic combinations; [-173A/-884T (AT), n=216; -173A/-884A (AA), n=27; -173T/-884T (TT), n=83; -173T/-884A (TA), n=24]. Box-plots depict the data where the box represents the interquartile range, the line through the box is the median, and whiskers illustrate the 10th and 90th percentiles. None of the genotypes at the -173 locus were significantly associated with altered levels of circulating IFN- α ($P=0.198$, ^aKruskal-Wallis test), while the presence of heterozygous (TA) genotype at -884, relative to homozygous wild-type (TT), was associated with significantly reduced levels of circulating IFN- α ($P=0.038$, ^bMann-Whitney U test). Presence of the -173T/-884A (TA) haplotypes was associated with reduced IFN- α levels compared to non-carriers ($P=0.031$, ^bMann-Whitney U test).

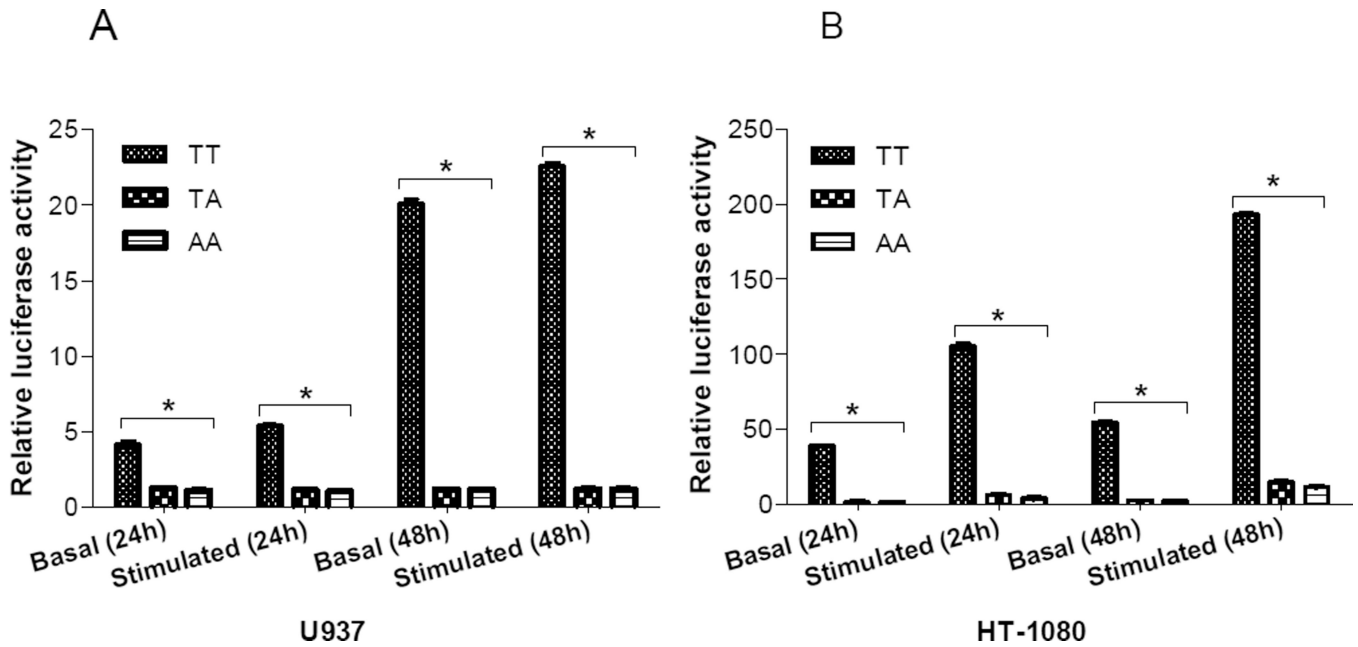


Figure 4. Analyses of IFNA8 promoter variants with luciferase reporter assays

The luciferase activity from test constructs, pGL3-basic, was measured and corrected for transfection efficiency with luciferase activity from the pGL3-control. Data are presented as the relative luciferase reporter activity, and each bar represents the mean \pm SEM of two replicate samples from three independent experiments with similar results. **(A)** U937 cells, P -values are calculated by Kruskal-Wallis test for across group corrected by Dunn's post tests ($*P=0.009$) and between two groups (TT vs. TA and TT vs. AA) by analysis of variance (Two-Way ANOVA) corrected by Bonferroni post tests ($P<0.001$). **(B)** HT-1080 cells, P -values are calculated by Kruskal-Wallis test for across group corrected by Dunn's post tests ($*P=0.021$) and between two groups (TT vs. TA and TT vs. AA) by two-way ANOVA corrected by Bonferroni post tests ($P<0.001$).

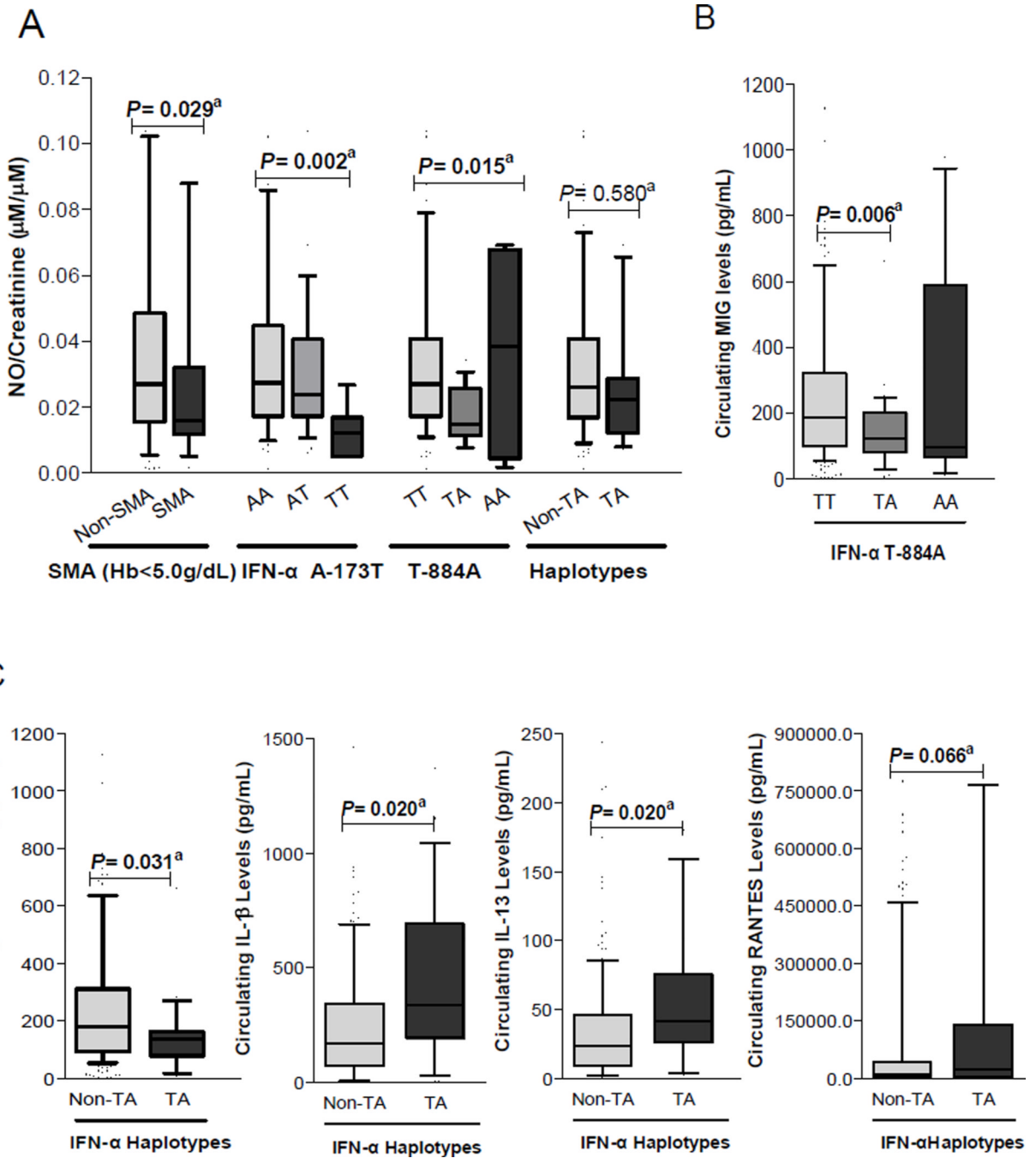


Figure 5. Production of systemic NO and inflammatory mediators

(A) Systemic urinary NO levels (NOx/creatinine in $\mu\text{M}/\mu\text{M}$) in children ($n=171$) aged <3.0 years with *P. falciparum* malaria were measured using the ‘Human Griess reaction’ (Anstey et al. 2002; Keller et al. 2004). Results are presented for each group as non-SMA (i.e., Hb >5.0 g/dL, $n=105$), SMA (i.e., Hb <5.0 g/dL, $n=66$), individual *IFNA2* and *IFNA8* promoter variants [A-173T (AA, $n=109$; AT, $n=48$; TT, $n=14$) and T-884A (TT, $n=142$; TA, $n=23$; AA, $n=6$)], and haplotypes [–173T/–884A (TA, $n=10$)]. Box-plots depict the data where the box represents the interquartile range, the line through the box is the median, and whiskers illustrate the 10th and 90th percentiles. As shown, significantly reduced NOx/

creatinine levels were found in SMA group compared to non-SMA group ($P=0.029$, ^bMann-Whitney U test), while the presence of TT genotype at -173 relative to homozygous wild-type (AA) was associated with significantly lower systemic NO_x/creatinine production ($P=0.002$, ^aMann-Whitney U test). Presence of TA genotype at -884 relative to homozygous wild-type (TT) was also associated with significantly lower NO_x/creatinine production ($P=0.015$, ^aMann-Whitney U test). Presence of the haplotype -173T/-884A (TA) was non-significantly associated with decreased NO_x/creatinine production compared to non-carriers ($P=0.580$ ^aMann-Whitney U tests). **(B & C)** Circulating MIG, IL-1 β , IL-13, and RANTES (pg/mL) in parasitemic children (n=243) were measured using the 'Human Cytokine Twenty-Five-Plex Antibody Bead Kit'. Results indicate that significantly reduced MIG levels were found in the TA genotype at -884 relative to homozygous wild-type (TT) ($P=0.006$, ^bMann-Whitney U test) and in the TA haplotype compared to non-carriers ($P=0.031$, ^aMann-Whitney U tests). Conversely, individuals with TA haplotype had elevated levels of IL-1 β , IL-13, and RANTES compared to non-carriers ($P=0.020$, $P=0.020$ and $P=0.066$, respectively, ^bMann-Whitney U test).

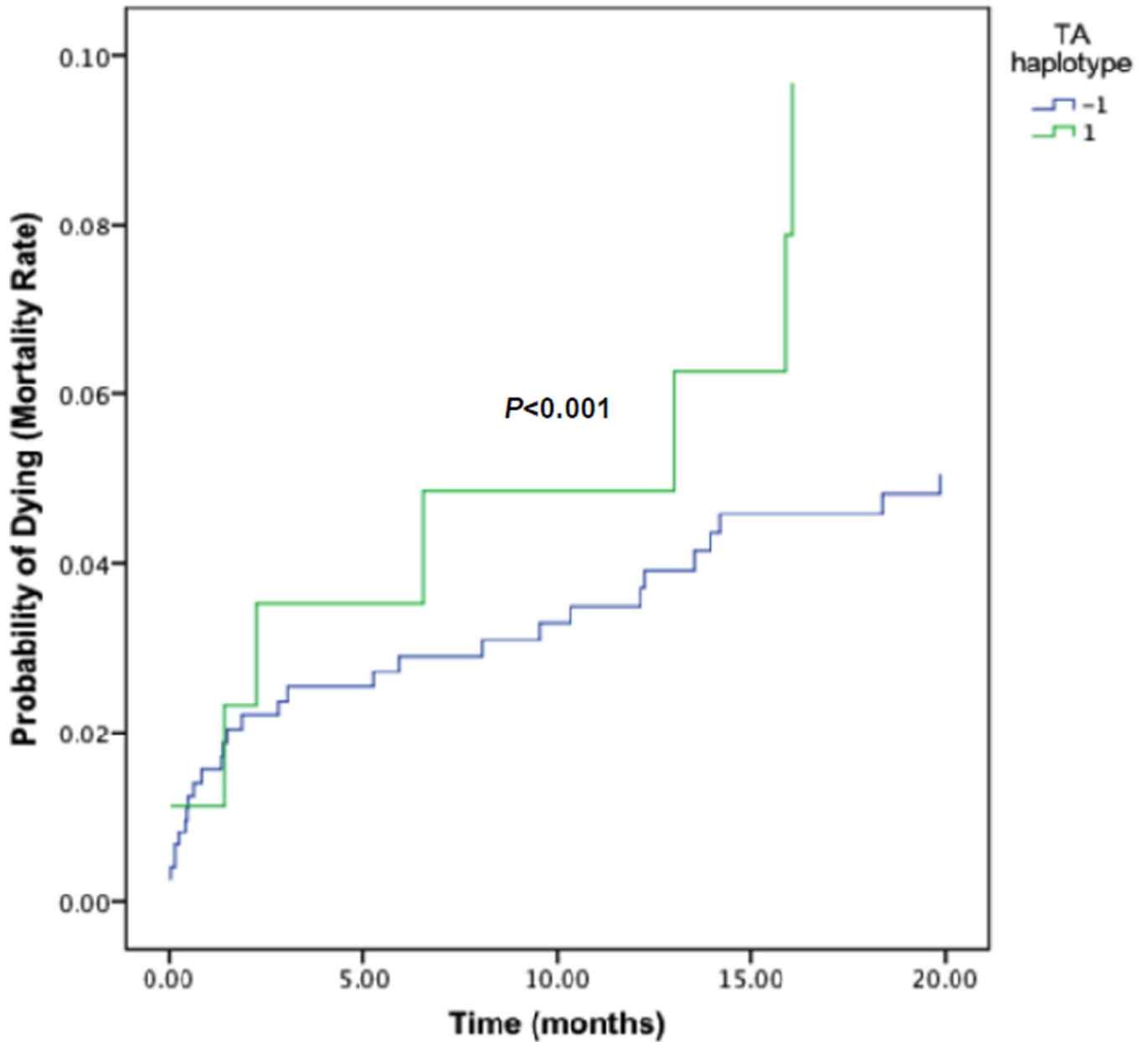


Figure 6. Probability of mortality in children stratified by haplotype carriage during the longitudinal study period

The probability of mortality rate was determined for the follow up period as a function of TA haplotype carriers (1; n=55) and non-carriers (-1; n=608). Green curve indicate TA carriers (1), and blue curve show non-carriers of TA haplotype (-1). Cox regression modeling revealed that mean hazard rates (the probability of dying over time) was significantly higher for carriers than non-carriers of the TA haplotype ($P < 0.001$).

Table 1

Demographic and laboratory characteristics of the study participants.

Characteristic	Non-SMA	SMA	P
No. of participants	384	124	N/A
Age, mos.	10.00 (9.00)	8.00 (9.75)	0.047^a
Gender, n (%)			
Male	196 (51.0)	58 (46.8)	0.235 ^b
Female	188 (49.0)	66 (53.2)	
Admission temp. (°C)	37.30 (1.60)	37.55 (1.68)	0.024^a
Hematological Indices			
Hemoglobin (g/dL)	7.00 (2.90)	4.20 (1.10)	<0.001^a
Hematocrit, %	21.90 (8.00)	13.00 (3.00)	<0.001^a
RBC count (10 ¹² /L)	3.37 (1.38)	1.81 (0.60)	<0.001^a
RDW	20.75 (5.40)	21.50 (4.70)	0.179 ^a
MCV, fL	68.75 (12.45)	73.40 (11.00)	<0.001^a
MCH, fL/cell	22.15 (4.00)	22.70 (5.00)	0.018^a
MCHC, g/dL	32.10 (2.00)	31.90 (4.00)	0.033^a
WBC count (10 ³ /μL)	11.30 (5.73)	15.00 (9.55)	<0.001^a
Lymphocytes, ×10 ³ /μL	5.50 (3.00)	6.90 (5.00)	<0.001^a
Monocytes, ×10 ³ /μL	1.00 (1.00)	1.60 (2.00)	<0.001^a
Granulocytes, ×10 ³ /μL	4.50 (4.00)	5.30 (5.00)	0.003 ^a
Platelets, ×10 ³ /μL	172 (162)	167 (138)	0.028 ^a
Reticulocyte Indices			
Absolute reticulocyte number (ARN)	66.00 (88.00)	46.00 (75.00)	0.005^a
Reticulocyte production index (RPI)	1.46 (2.00)	1.15 (1.70)	0.032^a
RPI<2, n (%)	259 (67)	100 (81)	0.008^b
Parasitological Indices			
Parasite density (μL)	19,718 (39,650)	16,728 (48,386)	0.468 ^a
HDP (n [%])	253 (66)	73 (59)	0.157 ^b
Genetic Variants			
Sickle cell trait, n (%)	50 (13)	8 (7)	0.127 ^b
G6PD deficiency, n (%)	14 (4)	6 (6)	0.541 ^b

Parasitemic children (n=508) were stratified into SMA (Hb<5.0 g/dL, with any density parasitemia); and non-SMA (Hb ≥ 5.0 g/dL, with any density parasitemia) (WHO 2000). Data presented are the median (interquartile range, IQR), unless otherwise stated. ARN and RPI were calculated, based on previous method (Were et al. 2009), as follows: reticulocyte index (RI) = (reticulocyte count × hematocrit) ÷ 30.7 (average hematocrit of children < 5 years of age in Siaya district); maturation factor (MF) = 1 + 0.05 (30.7 - hematocrit); RPI = RI/MF; ARN = (RI × RBC count /L) ÷

100. All subjects positive for HIV-1 or bacterial infections were excluded from the analyses. Abbreviations: N/A, not applicable; Hb - Hemoglobin; non-SMA - non-severe malarial anemia (Hb \geq 5.0g/dL, with any density parasitemia); SMA - severe malarial anemia (Hb < 5.0g/dL, with any density parasitemia); RDW - Red cell distribution width; MCV - Mean corpuscular volume; MCH - Mean corpuscular hemoglobin; MCHC - Mean corpuscular hemoglobin concentration; MPS - malaria parasites; HDP - high density parasitemia (MPS \geq 10,000/ μ L). RBC, red blood cell; WBC, white blood cell; HDP, high-density parasitemia \geq 10,000 parasites/ μ L.

^aStatistical significance determined by Mann-Whitney U test.

^bStatistical significance determined by the Chi-square (χ^2) analysis.

Table 2Distribution frequencies of *IFN* genotypes and haplotypes.

Genotype/Haplotype	Non-SMA	SMA	Total	<i>P</i>
No. of participants	384	124	508	
<i>IFNA2</i> (A-173T)				
AA,n(%)	235 (61.2)	78 (62.9)	313 (61.6)	0.814 ^a
AT,n(%)	119 (31.0)	35 (28.2)	154 (30.3)	
TT,n(%)	30 (7.8)	11 (8.9)	41 (8.1)	
Allele (A) frequency	0.77	0.77	0.77	
<i>IFNA8</i> (T-884A)				
TT,n(%)	306 (79.7)	108 (87.1)	414 (81.5)	0.080 ^a
TA,n(%)	62 (16.1)	10 (8.1)	72 (14.2)	
AA,n(%)	16 (4.2)	6 (4.8)	22 (4.3)	
Allele (T) frequency	0.88	0.91	0.89	
Haplotypes				
AT,n(%)	263 (68.6)	87 (70.3)	350 (69.0)	0.385 ^a
TT,n(%)	74 (19.2)	26 (20.8)	100 (19.6)	0.311 ^a
AA,n(%)	32 (8.3)	8 (6.7)	40 (7.9)	0.252 ^b
TA,n(%)	15 (3.9)	3 (2.2)	18 (3.5)	0.072 ^b

Data are n (%) unless otherwise indicated. *P*, major allele frequency; Statistical significance determined by the^aChi-square (χ^2) analysis and^bFisher's exact test.