Genetic variation in CSF2 (5q31.1) is associated with longitudinal susceptibility to pediatric malaria, severe malarial anemia, and all-cause mortality in a high-burden malaria and HIV region of Kenya

Lily E. Kisia1,2, Qiuying Cheng3, Evans Raballah2,4, Elly O. Munde4,5, Benjamin H. McMahon6, Nick W. Hengartner6, John M. Ong’echa7, Kiprotich Chelimo1, Christophe G. Lambert3, Collins Ouma1,2, Prakasha Kempalath8, Douglas J. Perkins2,3, Kristan A. Schneider9 and Samuel B. Anyona2,10*

Abstract
Plasmodium falciparum infections remain among the leading causes of morbidity and mortality in holoendemic transmission areas. Located within region 5q31.1, the colony-stimulating factor 2 gene (CSF2) encodes granulocyte–macrophage colony-stimulating factor (GM-CSF), a hematopoietic growth factor that mediates host immune responses. Since the effect of CSF2 variation on malaria pathogenesis remains unreported, we investigated the impact of two genetic variants in the 5q31.1 gene region flanking CSF2: g-7032 G > A (rs168681: G > A) and CSF2: g.64544T > C (rs246835: T > C) on the rate and timing of malaria and severe malarial anemia (SMA, Hb < 5.0 g/dL) episodes over 36 months of follow-up. Children (n = 1654, aged 2–70 months) were recruited from a holoendemic P. falciparum transmission area of western Kenya. Decreased incidence rate ratio (IRR) for malaria was conferred by inheritance of the CSF2: g.64544 TC genotype (P = 0.0277) and CSF2: AC/GC diplotype (P = 0.0015). Increased IRR for malaria was observed in carriers of the CSF2: AT/GC diplotype (P = 0.0237), while the inheritance of the CSF2 AT haplotype increased the IRR for SMA (P = 0.0166). A model estimating the longitudinal risk of malaria showed decreased hazard rates among CSF2 AC haplotype carriers (P = 0.0045). Investigation of all-cause mortality revealed that inheritance of the GA genotype at CSF2: g-7032 increased the risk of mortality (P = 0.0315). Higher risk of SMA and all-cause mortality were observed in younger children (P < 0.0001 and P = 0.0015), HIV-1(+) individuals (P < 0.0001 and P < 0.0001), and carriers of HbSS (P = 0.0342 and P = 0.0019). Results from this holoendemic P. falciparum area show that variation in gene region 5q31.1 influences susceptibility to malaria, SMA, and mortality, as does age, HIV-1 status, and inheritance of HbSS.

Keywords: P. falciparum, CSF2, GM-CSF, Malaria, Genotypes, Haplotypes, Diplotypes

Introduction
In 2020, the estimated number of malaria cases reported worldwide was 241 million [1]. A large proportion (95%; 228 million) of the cases occurred in the African region, mainly attributed to Plasmodium falciparum (P. falciparum) infections (> 99.8% of the total cases) [1]. Globally,
there were ~627,000 malaria-related deaths, for which the most vulnerable population were children under 5 years of age [1]. Accordingly, 77% of the global malaria mortalities were children residing in the World Health Organization (WHO) African region [1]. In western Kenya, a region holoendemic for *P. falciparum* transmission, malaria is one of the primary causes of childhood morbidity and mortality [2, 3]. In this region, severe *P. falciparum* infections manifest as severe malarial anemia [SMA, hemoglobin (Hb) < 5.0 g/dL] [4, 5]. Our previous investigations in the study area show that HIV-1 significantly increases the cross-sectional risk of SMA [6].

The pathophysiology of SMA is complex, and includes the destruction of malaria parasite-infected and non-infected erythrocytes, as well as decreased erythropoiesis [7]. We have demonstrated that variability in genes that encode immune-response proteins plays a key role in the pathogenesis of SMA, largely through imparting changes in soluble mediators of inflammation [8]. However, the causal molecular basis of the disease has not been fully elucidated.

Granulocyte monocyte-colony stimulating factor (GM-CSF) is a hematopoietic growth factor that facilitates the differentiation of progenitor cells into three lineages in the bone marrow, namely the lymphoid, myeloid, and erythroid progenies [9–11]. GM-CSF has been shown to promote growth and differentiation of leucocytes, and enhance release of other cytokines, which are central mediators of host immune responses [12, 13]. GM-CSF is secreted by an array of cell types including mast cells, B cells, activated T cells, fibroblasts, macrophages, vascular endothelial cells, and various oncogenic cells [9]. Secretion of GM-CSF is often accompanied by the release of additional inflammatory mediators, such as the granulocyte colony-stimulating factor (G-CSF) [10, 12], which in turn, modulate GM-CSF production through feedback regulatory mechanisms [14, 15].

The importance of GM-CSF in host immune response to both non-infectious and infectious diseases has been reported for tumor growth and metastasis, Crohn’s disease (CD), and tuberculosis [16–18]. With regard to malaria, studies utilizing murine models have reported: (1) a reduction in the levels of erythropoietic-related cytokines, including GM-CSF; (2) a negative correlation between GM-CSF concentrations and enhanced pathology in malarial anemia; and (3) elevated levels of GM-CSF in lethal malaria [19, 20]. In the context of human malaria, the toll-like receptors (TLR) 7/8 stimulated production of GM-CSF was elevated in cord blood cells of infants with evidence of past placental malaria [21], suggesting a profound effect on the fetal immune system, with the differential alternations in innate immune responses predicting the risk of malaria during the first year of life [21]. Moreover, elevated serum levels of GM-CSF have been reported in cases of severe *P. falciparum* malaria [22]. Previous investigations in our laboratories identified elevated levels of GM-CSF in children with SMA compared to those with non-SMA, and elevated GM-CSF levels in children with *P. falciparum* and HIV-1 co-infection relative to children with malaria alone [23, 24].

The gene that encodes GM-CSF is colony-stimulating factor 2 (*CSF2*), which is located on the human chromosome at 5q23-31, in close proximity to interleukin 3 (*IL3*) within a T helper type 2-associated gene cluster [25]. *CSF2* spans ~2.5 kb in length, and encompasses 4 exons and 3 introns [26]. *CSF2* expression is regulated at both the transcriptional and post-transcriptional stages [27–29]. Elements in the proximal promoter of *CSF2* were shown to contribute to transcriptional regulation of *CSF2* by multiple transcription factors such as the AP-1, ZEB1, NF-AT, and GATA [27–29]. At the post-transcriptional regulation level, a stretch of AU-rich elements (ARE) in the 3′ untranslated region (3′-UTR) of *CSF2* mRNA mediates the binding of ARE-binding proteins to *CSF2* mRNA, causing degradation of this transcript [28, 29]. Interestingly, various distal enhancers were shown to modulate transcription of human target genes such as *CCR5*, *BRN3A*, *CDKN1C*, *VEGFA*, *ADGB*, *C-myb*, *SOX-9*, *SCNA*, *C-myc*, *cPLA2α* gene (*PLA2G4A*) and renin gene (*REN*) by forming cognate enhancer–promoter loops with the target gene promoter site [30–36]. Some of these distal enhancers are located at varied distances upstream of the transcription start site (TSS) of the corresponding gene: ~552 kb for *CCR5*, ~55 kb for *BRN3A*, over 100 kb for *CDKN1C*, ~157 kb for *VEGFA*, ~34 kb for *C-myb*, ~635 kb for *SOX-9*, ~9.5 kb for *PLA2G4A*, and ~5.3 kb for *REN* [31, 32, 34, 35]. In contrast, other distal enhancers are located at varied distances downstream of corresponding TSS of the gene: 216 kb for *ADGB*, 85 kb for *SCNA*, and 1.43 Mb for *C-myc* [33, 36]. Collectively, these studies show that the enhancers can be located either up- or downstream at varied distances of target genes.

A number of studies have found associations between polymorphic variability in the *CSF2* gene and outcomes of various disease [37–42], while other studies observed no relationships [43, 44]. In order to understand the influence of *CSF2* variants on susceptibility to malaria and SMA, the current study investigated the impact of two SNPs flanking the *CSF2*-g.-7032 G > A (rs168681:G > A) and *CSF2*-g.64544T>C (rs246835:T>C). The first SNP, rs168681:G>A is about 6 kb upstream of the 1 kb proximal promoter of *CSF2* and has recently emerged among the genetic biomarkers for prediction of the urinary nicotine metabolite ratio in multiethnic samples [45].
The second SNP, rs246835:T>C has not been linked to any diseases. We selected these two SNPs based on their characteristics of (1) minor allele frequencies (MAF) ≥10% in African populations and (2) the ability to impart functional changes in transcription-factor binding sites (TFBS) within corresponding distal enhancers [27, 28]. The relationship between the SNPs (and their haplotypes and diplotypes) on susceptibility to malaria, SMA and mortality were investigated longitudinally over a 36-month follow-up in a cohort of children (n=1654; age 2–70 months at enrollment) resident in a P. falciparum holoendemic region of Kenya.

**Methods**

**Study area**

This study was conducted at Siaya County Referral Hospital (SCRH), located in a holoendemic P. falciparum transmission area in western Kenya. Details of the study site have previously been published [46]. One of the primary causes of childhood mortality and morbidity in the Siaya community is P. falciparum malaria [47], whose transmission has remained stable, despite malaria control efforts [48]. SMA accounts for one of the highest percentages of hospital-bed occupancies in SCRH, and results in significant in-hospital morbidity and mortality [4]. Individuals inhabiting the study area are predominantly from the Luo ethnic group (>96%), a culturally homogeneous population [49].

**Study participants**

Children presenting with suspected malaria infections and those reporting for routine vaccinations were recruited at SCRH. After screening for malaria parasites, children (aged 2–70 months) with varying severities of malarial anemia (n=1319) and aparasitemic controls (n=335) were enrolled. Children were excluded from the study if they presented with non-falciparum parasite strains, had confirmed cerebral malaria, were previously hospitalized for any reason, or had reported use of anti-malarial therapy in the two preceding weeks. The current study utilized two cohorts recruited and followed with identical parameters across a temporal continuum: cohort 1 (2003–2005; n=777) and cohort 2 (2007–2012; n=877).

**Longitudinal follow-ups**

After enrollment (Day 0), children (n=1654) were scheduled for follow-up visits on day 14 (if they were febrile upon enrollment) and quarterly over 36 months [50–52]. Parents/guardians who failed to return for scheduled quarterly follow-up visits were traced by the study team at their residence to check the child's health status, including mortality. Each residence was identified by a GIS/GPS surveillance system. In addition, parents/guardians were asked to return to the hospital any time their child was febrile [acute febrile episode(s)]. Physical evaluations and laboratory tests required for comprehensive clinical management of the patients were performed at enrollment, day 14, and each acute and quarterly visit (complete blood counts [CBC], malaria parasitemia measures, and evaluation of bacteremia where indicated). For all acute episodes and scheduled visits, children were managed according to the Ministry of Health-Kenya guidelines.

**Laboratory procedures**

Heel and/or finger-prick blood samples (<100 µL) and venipuncture blood (1–3 mL) were obtained and used to determine parasite densities. Giemsa-stained thin blood smears were prepared, examined, and asexual malaria parasite densities determined as previously published [46]. Complete blood counts (CBCs) were determined using a Beckman-Coulter AcT diff2 (Beckman-Coulter Corporation, Brea, CA, USA). At enrollment, based on Hb concentrations, children with any density parasitemia, were stratified into either non-SMA (Hb ≥5.0 g/dL; n=1029) or SMA (Hb<5.0 g/dL; n=290), according to the WHO definition of SMA [53]. Aparasitemic children (n=335; P. falciparum negative blood smear), recruited from vaccination clinics at SCRH, were age and gender-matched.

Since co-infections influence the severity of malarial anemia in Siaya, all children were tested for HIV-1 and bacterial infections as previously described [6, 54]. Parents/legal guardians of participating children received pre- and post-test HIV&AIDS counseling. At the time of enrollment, none of the children were on antiretroviral therapy for HIV-1.

To further discern chronic anemia resulting from genetic factors, sickle cell traits and α3.7-thalassemia deletions were investigated. Sickle cell status was determined using the alkaline cellulose acetate electrophoresis on Titan III plates (Helena BioSciences, Sunderland, UK). The α3.7-thalassemia deletion variants were determined as previously described [55].

**CSF2 genotyping**

The BuccalAmp™ DNA extraction kit (Epicentre Biotechnologies, Madison, WI, USA) was used to extract genomic DNA from buccal swabs collected from each of the 1654 study participants described above. The resulting genomic DNA was then amplified using the Genomi phi DNA amplification kit (GE Healthcare, Life Sciences, Marlborough, MA, USA), according to the manufacturer’s instructions. Genetic variants rs168681:G>A and rs246835:T>C were genotyped using the TaqMan
5′ allelic discrimination Assay-By-Design (assay IDs C_3285157_20 and C_2397167_10, respectively). Allelic discrimination was performed using the StepOne™ Software Version 2.3 (Thermo Fisher Scientific, Carlsbad, CA, USA).

Amplification was conducted in a total reaction volume of 20 μL, with the following conditions: initial denaturation at 60 °C for 30 s and 95 °C for 10 min., followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min., and a final extension (60 °C for 30 s). To validate results obtained with the Taqman® real-time genotyping assays, 10% of the samples were randomly selected and genotyped using restriction fragment length polymorphism PCR. There was 100% agreement between the two methods.

Data analyses
Data were analyzed using R version 3.1.4 [56]. Data from both cohorts (1: 2003–2005, n = 777, and 2: 2007–2012, n = 877) were combined into one data set. However, cohort was kept as a categorical covariate to account for potential changing malaria incidence across time. Metric variables upon enrollment were compared across aparasitemic, non-SMA, and SMA patients. For metric variables, boxed plots, histograms, and Q–Q-plots were used to identify normally distributed variables. The median (interquartile range) and mean (standard deviation) were calculated for each variable. For normally distributed variables, one-way ANOVAs and two-sample T-tests were used. If normality was violated, Kruskal–Wallis tests and Mann–Whitney U tests were used. The distribution of categorical variables was compared between the groups (aparasitemic, non-SMA, and SMA) using a Chi-square test for homogeneity. If cell count was < 20, a generalized Fisher's exact test was performed. If numerically not feasible, P-values were approximated using a Monte Carlo method, with B = 50,000 contingency tables.

Haplotypes and diplotypes were constructed to investigate the effects of the genetic architecture determined by the CSF2:g.-7032 G > A (rs168681:G > A) and CSF2:g.64544T>C (rs246835:T>C) loci. The top-level was the individual diplotypes, consisting of 2 of the 4 possible haplotypes; GT, GC, AT, and AC. At the marginal levels, the diplotypes gave rise to the (1-locus) genotypes at CSF2:g.-7032 G > A (GG, GA, AA) and CSF2:g.64544T>C (TT, TC, CC). For all generalized linear and hazard models performed, the effects of the genetic architecture were coded as 0/1 variables that reflected: (1) per locus contributions determined by the mutant homozygotes and heterozygotes (indicating additive and dominant effects), (2) haplotype contributions (reflecting epistatic interactions), and (3) diplotype contributions (reflecting positional effects). The respective wild types were subsumed as a baseline factor. Hence, we coded the following 0/1 variables: (1) genotypes GA, AA for CSF2:g.-7032 G > A and TC, CC for CSF2:g.64544T > C; (2) haplotypes GC, AT, AC; and (3) diplotypes GT/GC, GT/AT, GT/AC, GC/GC, GC/AC, AT/AT, AT/AC, AC/AC. We tested for differences in the frequency distributions between the aparasitemic, non-SMA, and SMA groups with the generalized Fisher’s exact test with simulated P-values. Exact tests for deviations from Hardy–Weinberg equilibrium (HWE) at the rs168681:G > A and the rs246835:T>C loci were performed as previously described [57]. Furthermore, due to low cell counts for some genotypes, Fisher’s exact test was performed to estimate departures from linkage-equilibrium between the two SNPs. Linkage disequilibrium (LD) of these two SNPs was determined by HaploView (version 4.2, Broad Institute, Cambridge, MA, USA).

The effect of covariates on the rate of malaria and SMA episodes was investigated using Poisson regression, with the model of best fit determined using Akaike’s information criterion (AIC). CSF2 genetic variants, age, sex, cohort, hemoglobinopathies (sickle cell traits and α3.7-thalassemia deletions), and co-infections (HIV-1 and bacteremia) were included as covariates. Only co-variables present in more than two samples were retained in the model.

An ordered multiple-outcome-per-subject Cox proportional hazard model was used to investigate the longitudinal influence of covariates (as listed above) on the occurrence of malaria and SMA episodes. Independent increments were used according to Anderson–Gill and data were right-censored to account for cases in which a patient was malaria or SMA free upon clinical presentation.

The influence of covariates on the risk of mortality was investigated using a Cox proportional hazard model. For each patient, either mortality or survival occurred at the last hospital visit. The last recorded hospital visit was the time to the event, or censoring, in the case of survival. Age (at the last hospital visit) was included as a covariate. The model was fit using all-cause mortality as the outcome variable.

Results
Demographic, clinical, and laboratory characteristics
Children (n = 1654) were categorized into aparasitemic (n = 335), and parasitemic (n = 1319) stratified into non-severe malarial anemia (non-SMA, hemoglobin; Hb ≥ 5.0 g/dL, n = 1029), and severe malarial anemia (SMA, Hb < 5.0 g/dL, n = 290) groups. The demographic, clinical, and laboratory characteristics of study participants are presented in Table 1. The distribution of sex was comparable (P = 0.6991) across the groups. Although the distribution of SMA cases were equal in
cohort 1 (2003–2005) and cohort 2 (2007–2012), they differed significantly between aparasitemic and non-SMA groups \((P = 5.5311 \times 10^{-11})\). Children with SMA were younger \((P = 0.0152)\) relative to aparasitemic and non-SMA groups, but the difference was not significant after correction for multiple testing. Axillary temperature differed across the groups and was higher in children with non-SMA \((P = 1.4420 \times 10^{-14})\), compared to those with parasitemia or SMA. Children were stratified a priori based on Hb concentrations. As such, relative to both the aparasitemic and non-SMA groups, children with SMA presented with significantly decreased hematocrit levels \((P = 1.4732 \times 10^{-174})\). White blood cell counts progressively increased across the groups \((P = 4.3233 \times 10^{-12})\), as did the mean corpuscular volume \((P = 7.9395 \times 10^{-21})\), with the SMA group having the highest levels (Table 1). Parasite densities were lower in children with SMA relative to non-SMA \((P = 2.9626 \times 10^{-25})\). The presence of HIV-1 did not significantly differ across groups \((P = 0.0729)\), nor did bacteremia status \((P = 0.1583)\). However, the distribution of sickle cell genotypes differed across groups \((P = 1.9990 \times 10^{-06})\), with a lower frequency of HbAS carriage in children with non-SMA and lowest frequencies among children with SMA. The distribution of α3.7-thalassemia deletion variants were also different across

**Table 1** Demographic, clinical, and laboratory characteristics of the study participants

| Characteristics                          | Total | Aparasitemic (MPS negative) | Non-SMA (Hb ≥ 5.0 g/dL) | SMA (Hb < 5.0 g/dL) | \(P\) |
|------------------------------------------|-------|-----------------------------|-------------------------|---------------------|------|
| Number of participants                   | 1654  | 335                         | 1029                    | 290                 |      |
| Sex [n, (%)]                             |       |                             |                         |                     |      |
| Female                                   | 1654  | 822 (49.70)                 | 168 (50.15)             | 504 (48.98)         | 150 (51.72) | 0.6991\(^a\) |
| Male                                     | 832   | (50.30)                     | 167 (49.85)             | 525 (51.02)         | 140 (48.28) |      |
| Study cohort [n, (%)]                    |       |                             |                         |                     |      |
| One (1)                                  | 1654  | 777 (46.98)                 | 209 (62.39)             | 423 (41.11)         | 145 (50.00) | 5.5311 \times 10^{-11}\(^a\) |
| Two (2)                                  | 877   | (53.02)                     | 126 (37.61)             | 606 (58.89)         | 145 (50.00) |      |
| Age, months                              | 1651  | 13.85 (8.14)                | 13.82 (8.67)            | 14.20 (7.88)        | 12.64 (8.34) | 0.0152\(^b\) |
| Axillary temperature, °C                 | 1636  | 37.67 (1.06)                | 37.27 (1.00)            | 37.80 (1.09)        | 37.69 (0.89) | 1.442 \times 10^{-14}\(^a\) |
| Hematological and parasitological parameters |       |                             |                         |                     |      |
| Hemoglobin, g/dL                         | 1639  | 7.47 (2.51)                 | 9.40 (2.63)             | 7.79 (1.82)         | 4.13 (0.73) | NA    |
| Hematocrit, %                            | 1609  | 24.34 (7.72)                | 29.52 (80)              | 25.40 (5.76)        | 14.22 (3.88) | 1.4732 \times 10^{-14}\(^b\) |
| White blood cells, \(\times 10^3/\mu L\) | 1602  | 13.40 (6.98)                | 13.06 (8.11)            | 12.77 (5.52)        | 16.16 (9.39) | 4.3233 \times 10^{-12}\(^b\) |
| Mean corpuscular volume, fl              | 1603  | 70.10 (9.30)                | 69.87 (8.41)            | 68.89 (8.48)        | 74.90 (11.46) | 7.9395 \times 10^{-21}\(^b\) |
| Parasite density, MPS/µL                 | 1644  | 55,716.67 (111,089.67)      | 0.00 (0.00)             | 72,983.96 (124,507.30) | 59,082.88 (103,946.74) | 2.9626 \times 10^{-20}\(^b\) |
| Endemic co-infections                    |       |                             |                         |                     |      |
| HIV1, [n, (%)]                           |       |                             |                         |                     |      |
| Negative (0)                             | 1641  | 1573 (95.86)                | 315 (96.04)             | 990 (96.49)         | 268 (93.38) | 0.0729\(^c\) |
| Positive (1)                             | 68    | (4.14)                      | 13 (3.96)               | 36 (3.51)           | 19 (6.62) |      |
| Bacteremia                               |       |                             |                         |                     |      |
| Negative (0)                             | 1639  | 1518 (92.62)                | 295 (90.77)             | 960 (93.57)         | 263 (91.32) | 0.1583\(^a\) |
| Positive (1)                             | 121   | (7.38)                      | 30 (9.23)               | 66 (6.43)           | 25 (8.68) |      |
| Genetic variants                         |       |                             |                         |                     |      |
| Sickle cell trait, n (%)                 |       |                             |                         |                     |      |
| Hb AA                                    | 1619  | 1358 (83.88)                | 252 (78.26)             | 848 (83.38)         | 258 (92.14) | 1.9990 \times 10^{-06}\(^d\) |
| Hb AS                                    | 243   | (15.01)                     | 60 (18.63)              | 165 (16.22)         | 18 (6.43) |      |
| Hb SS                                    | 18    | (1.11)                      | 10 (3.11)               | 4 (0.39)            | 4 (1.43) |      |
| \(\alpha^+\)-Thalassemia deletion, n (%) |       |                             |                         |                     |      |
| \(\alpha\alpha\)/\(\alpha\alpha\)       | 1405  | 596 (42.42)                 | 120 (43.96)             | 375 (42.86)         | 101 (39.30) | 0.0433\(^a\) |
| \(-\alpha^+/-\alpha\)                    | 526   | (37.44)                     | 84 (30.77)              | 335 (38.29)         | 107 (41.63) |      |
| \(-\alpha^+/-\alpha^+\)                  | 283   | (20.14)                     | 69 (25.27)              | 165 (18.86)         | 49 (19.07) |      |

Data are presented as mean (standard deviation). Children \((n = 1654)\) were categorized into aparasitemic controls \((n = 335)\; no MPS\) and according to the WHO definition of SMA \([53]\), into either non-SMA \((n = 1029; \text{Hb} \geq 5.0 \text{ g/dL})\) or SMA \((n = 290; \text{Hb} < 5.0 \text{ g/dL})\). MPS malaria parasites, HIV human immunodeficiency virus.

\(^a\)Pearson’s Chi-squared test; \(^b\)Student’s t test; \(^c\)Fisher’s exact test for count data; \(^d\)Fisher’s exact test for count data with simulated \(P\)-value (based on 5e+05 replicates)
the groups \(P=0.0433\), with the highest frequencies of single and double-deletions in children SMA, however the difference is not significant after correction for multiple testing.

**Linkage disequilibrium**

The location of the two SNPs selected for investigation in the CSF2 gene region (5q31.1) [58] is shown in Fig. 1a and b. The chromosomal location of rs168681:G > A is 5:132066757, 7032 bp upstream of TSS of CSF2, and 1279 bp downstream of LOC108449898. The chromosomal location of rs246835:T > C SNP is 5:132138332, 64544 bp downstream of the TSS of CSF2, 34642 bp downstream of LOC112997558, and 46544 bp upstream of the human P4HA2-AS1 gene [58] (Fig. 1a, b).

Linkage disequilibrium analysis revealed a weak association between the two CSF2 variants (\(D^2=0.416, \text{LOD}=4.32, r^2=0.01\); Fig. 1c). Departures from linkage equilibrium (LE) were significant for the overall sample and stratified by aparasitemic, non-SMA, and SMA groups. However, the departure from LE was not significant in the SMA group after multiple testing, suggesting that these departures are marginal and only identified as significant due to the large sample size.

**Selection of CSF2 SNPs**

As recently described [50], a pilot high-throughput genotyping experiment was performed using the Human Immunochip® (coated with > 196 K markers, Illumina®, San Diego, CA, USA) in a subset of children representing “polarized extremes” of severe and non-severe malaria: cases (SMA with Hb < 5.0 g/dL, \(n=70\)) vs. controls (non-SMA with Hb > 8.0 g/dL, \(n=74\)). Immunochip® data were analyzed using logistic regression analysis, with an additive mode of inheritance and identified an intergenic SNP rs246835:T > C within the CSF2 haplotype that was associated with increased risk to SMA [odds ratio (OR) = 2.771, 95% confidence interval (CI) = 0.904–8.494, \(P=0.059\)]. We selected rs246835:T > C since this SNP is part of a potential distal enhancer with the loss of TFBS for USF2 and creation of TFBS for GATA-1 by switch of T allele to C allele (Fig. 1d). rs246835:T > C had a minor allele frequency (MAF) of 0.10 in the African (AFR) population (1000 Genome Project), and a MAF of 0.14 in the Yoruban (YRI) population (HapMap) (Fig. 1d). Though not in the Immunochip®, SNP rs168681:G > A had desirable characteristics for further exploration, including: (1) a MAF of 0.20 (1000 Genome Project) in the AFR population and a MAF of 0.18 in the YRI population (HapMap), and (2) potential functional effects, where the wild-type G allele creates TFBS for ER-alpha and ZEB1, and transition to the minor A allele ablates such binding and creating a TFBS for NF-X3. These TFBSs are part of a potential distal enhancers (Fig. 1d).

**Distribution of the CSF2 genotypes, haplotypes, and diplotypes**

Following genotyping, SNP calling was successful for rs168681:G > A and rs246835:T > C variants, with both genotypes present in 1203 out of 1654 study participants recruited into cohorts 1 and 2. To increase the power of analysis, no imputations were performed to infer genotypes, haplotypes or diplotypes. As such, only data with both SNPS present for each participant were utilized for subsequent statistical analyses (Table 2).

The distribution of CSF2 genotypes, haplotypes, and diplotypes in aparasitemic, non-SMA, and SMA groups is presented in Table 2. The genotypic proportions for rs168681:G > A (\(P=0.9972\)), and rs246835:T > C (\(P=0.2202\)) were comparable across the groups. The allele frequencies for the study population is comparable to those from the International HapMap Project and 1000 Genomes Project (Fig. 1d). The HWE analyses showed a departure for the rs246835:T > C for the overall population, and in the aparasitemic, non-SMA, and SMA groups. There was no departure from HWE for the rs168681:G > A polymorphic variant (Table 2). The distribution of haplotypes (\(P=0.5565\)), and diplotypes (\(P=0.3255\)) were not significantly different across the study groups.

**Relationship between CSF2 variants and the rate of malaria episodes**

In malaria holoendemic regions, such as western Kenya, children experience multiple episodes of malaria from infancy onward, with occasional life-threatening complications of SMA before the development of naturally acquired malaria immunity [7]. We, therefore, investigated the impact of the CSF2 variants on malaria episodes over a follow-up period of 36 months. Factors
**Fig. 1** (See legend on previous page.)

### Table: CSF2 Alleles and TFBS

| Alleles | Population | 1000 Genome | HapMap | TFBS          |
|---------|------------|-------------|--------|--------------|
|         | AFR        | YRI         |        |              |
| rs168681:G>A |
| Alleles | G          | 0.80        | 0.82   | ER-alpha, ZEB1 |
|         | A          | 0.20        | 0.18   | NF-X3        |
| rs246835:T>C  |
| Alleles | T          | 0.90        | 0.86   | USF2         |
|         | C          | 0.10        | 0.14   | GATA-1       |
The rate of malaria episodes were determined using a generalized linear model (Poisson regression). Results for the covariates that emerged for the 6029 recorded malaria episodes over 36 months of follow-up are shown in Table 3. Age at enrollment was an important variable for the rate of malaria episodes, with older children presenting a decreased risk over time (incidence rate ratio (IRR; for a delta of 1 month) = 0.967 (95% CI 0.963--0.970), P<0.0001). Children who were HIV-1 positive had a lower incidence rate of malaria episodes (P=0.0023), relative to HIV-1 negative participants, as did female study participants (P=0.0013), compared to their male counterparts. Carriage of the homozygous double deletion (−α3.7/−α3.7) for α+-thalassemia also conferred a protective effect against malaria episodes (P=0.0068) relative to homozygous αα/αα carriers. Protection against multiple bouts of malaria over time was also associated with co-inheritance of homozygosity for the sickle cell disease (Hb SS, P<0.0001) and heterozygosity for the sickle cell trait [Hb AS, (P<0.0001). Protective effects against the longitudinal risk of malaria were also present in heterozygous carriers of the rs246835 TC genotype (P=0.0277) relative to the wild type genotype TT. Similarly, the positional interaction of the CSF2 AC/GC diplotype was associated with decreased malaria episodes (P=0.0015) compared to wild-type diplotype GT/GT.

Table 2  Distribution of CSF2 genotypes, haplotypes, and diplotypes

| Variants | Total | Aparasitemic (MPS negative) | Non-SMA (Hb ≥ 5.0 g/dL) | SMA (Hb < 5.0 g/dL) | P value |
|----------|-------|-----------------------------|-------------------------|---------------------|---------|
| No. of participants | 1203 | 250 | 772 | 181 | |
| CSF2g.-7032 G > A (rs168681:G > A) genotype | | | | | |
| GG | 636 (52.87) | 134 (53.60) | 407 (52.72) | 95 (52.49) | 0.9972a |
| GA | 476 (39.57) | 98 (39.20) | 305 (39.51) | 73 (40.33) | |
| AA | 91 (7.56) | 18 (7.20) | 60 (7.77) | 13 (7.18) | |
| P value | 0.8847 | 1.0000 | 0.7869 | 1.0000 | |
| Allele frequency (p/q) | 0.727/0.273 | 0.732/0.268 | 0.725/0.275 | 0.726/0.274 | |
| HWE, P value | 0.8847 | 1.0000 | 0.7869 | 1.0000 | |
| CSF2g.64544T > C (rs246835:T > C) genotype | | | | | |
| TT | 984 (81.80) | 196 (78.40) | 636 (82.38) | 152 (83.98) | 0.2202a |
| TC | 125 (10.39) | 35 (14.00) | 77 (9.97) | 13 (7.18) | |
| CC | 94 (7.81) | 19 (7.60) | 59 (7.64) | 16 (8.84) | |
| P value | 2.6077 × 10−55 | 2.0014 × 10−59 | 1.9886 × 10−36 | 2.6515 × 10−13 | |
| Allele frequency (p/q) | 0.870/0.130 | 0.854/0.146 | 0.874/0.126 | 0.876/0.124 | |
| HWE, P value | 2.6182 × 10−55 | 2.0014 × 10−9 | 1.9896 × 10−36 | 2.6528 × 10−13 | |
| CSF2g.-7032 G > A (rs168681:G > A)/CSF2g.64544T > C (rs246835:T > C) haplotypes | | | | | |
| GT | 1470 (61.10) | 297 (59.40) | 949 (61.50) | 224 (61.90) | 0.5565a |
| GC | 278 (11.60) | 69 (13.80) | 170 (11.00) | 39 (10.80) | |
| AT | 623 (25.90) | 130 (26.00) | 400 (25.90) | 93 (25.70) | |
| AC | 35 (1.50) | 4 (0.80) | 25 (1.60) | 6 (1.70) | |
| LE, P value | 1.6781 × 10−13 | 1.2543 × 10−36 | 2.0835 × 10−57 | 0.0307 | |
| CSF2g.-7032 G > A (rs168681:G > A)/CSF2g.64544T > C (rs246835:T > C) diplotypes | | | | | |
| GT/GT | 500 (41.56) | 93 (37.20) | 331 (42.88) | 76 (41.99) | 0.3255a |
| GT/GC | 71 (5.90) | 15 (10.00) | 38 (4.92) | 8 (4.42) | |
| GC/GC | 65 (5.40) | 16 (6.40) | 38 (4.92) | 11 (6.08) | |
| GT/AT | 399 (33.17) | 86 (34.40) | 249 (32.25) | 64 (35.36) | |
| GT/AC | 27 (2.24) | 3 (1.20) | 19 (2.46) | 5 (2.76) | |
| GC/AC | 85 (7.07) | 17 (6.80) | 56 (7.25) | 12 (6.63) | |
| AT/AT | 50 (4.16) | 9 (3.60) | 37 (4.79) | 4 (2.21) | |
| AT/AC | 4 (0.33) | 1 (0.40) | 2 (0.26) | 1 (0.55) | |
| AC/AC | 2 (0.17) | 0 (0.00) | 2 (0.26) | 0 (0.00) | |

Data are presented as proportions [n, (%)] unless otherwise stated. Children (n = 1203) were categorized into aparasitemic controls (n = 250; no parasitemia) and according to the WHO definition of SMA [53], into either non-SMA (n = 772; Hb ≥ 5.0 g/dL) or SMA (n = 181; Hb < 5.0 g/dL). Fisher’s exact test with simulated P-values.

MPS malaria parasites, Hb hemoglobin, CSF2 colony-stimulating factor 2, p major allele, q minor allele, HWE Hardy–Weinberg Equilibrium, LE linkage equilibrium.
Conversely, co-inheritance of the CSF2 AT/GC diplotype increased the rate of malaria episodes ($P = 0.0237$). There was also a trend towards more malaria episodes in carriers of the CSF2 GC/GT diplotype, however, the trend was not significant ($P = 0.0663$) (Table 3).

**Relationship between CSF2 variants and the rate of SMA episodes**

The effect of the CSF2 variants on the rate of SMA episodes over a follow-up period of 36 months was investigated using a Poisson regression as described above. Factors associated with the rate of SMA for the 297 episodes that occurred over the 36 months of follow-up are shown in Table 3. Consistent with the results observed for susceptibility to malaria, children who were older at admission into the study had decreased incidence rates for SMA ($P < 0.0001$). Similarly, children who were recruited into cohort 2 (2007–2012) had lower incidences of SMA ($P = 0.0013$), relative to those enrolled in study cohort 1 (2003–2005). Unlike the protection observed against malaria in HIV-1-positive children, being positive for HIV-1 markedly increased the risk of SMA ($P < 0.0001$). Inheritance of the double mutation that confers sickle cell disease (Hb SS) increased the rate of SMA ($P = 0.0024$), whereas carriage of sickle cell trait (Hb AS) protected against SMA episodes ($P = 0.0026$). The only CSF2 variant found to influence the rate of SMA episodes was carriage of the

| Variable names | Event | Baseline | Estimate | Std. error | z value | IRR (95% CI) | P value |
|----------------|-------|----------|----------|------------|---------|--------------|---------|
| Malaria episodes | (Intercept) | —1.389 | 0.032 | —43.218 | 0.249 (0.234–0.265) | <0.0001 |
| Age at enrollment | —0.034 | 0.002 | —18.123 | 0.967 (0.963–0.970) | <0.0001 |
| Cohort* | 499 | 576 | 0.044 | 0.029 | 1.509 | 1.045 (0.987–1.106) | 0.1313 |
| HIV1 (+) | 36 | 1039 | —0.318 | 0.104 | —3.044 | 0.728 (0.593–0.989) | 0.0023 |
| Sex (female) | 540 | 535 | —0.089 | 0.028 | —3.225 | 0.915 (0.867–0.966) | 0.0013 |
| -α[3.7]/-α[3.7] | 215 | 860 | —0.097 | 0.036 | —2.709 | 0.908 (0.846–0.974) | 0.0668 |
| Hb SS | 14 | 1061 | —0.750 | 0.181 | —4.155 | 0.473 (0.332–0.673) | <0.0001 |
| Hb AS | 159 | 916 | —0.228 | 0.042 | —5.455 | 0.796 (0.734–0.864) | <0.0001 |
| CSF2:g.64544 TC genotype | 115 | 960 | —1.102 | 0.501 | —2.201 | 0.332 (0.125–0.886) | 0.0277 |
| CSF2 GC/GT diplotype | 66 | 1009 | 0.926 | 0.504 | 1.837 | 2.525 (0.940–6.787) | 0.0663 |
| CSF2 AT/GC diplotype | 46 | 1029 | 1.142 | 0.505 | 2.262 | 3.132 (1.164–8.422) | 0.0237 |
| CSF2 AC/GC diplotype | 25 | 1050 | —0.313 | 0.099 | —3.174 | 0.732 (0.603–0.887) | 0.0015 |
| SMA episodes | (Intercept) | —4.147 | 0.132 | —31.452 | 0.016 (0.012–0.021) | <0.0001 |
| Age at enrollment | —0.056 | 0.009 | —6.118 | 0.946 (0.929–0.963) | <0.0001 |
| Cohort* | 499 | 576 | —0.441 | 0.137 | —3.222 | 0.644 (0.492–0.841) | 0.0013 |
| HIV1 (+) | 36 | 1039 | 1.060 | 0.239 | 4.431 | 2.887 (1.806–4.614) | <0.0001 |
| Hb SS | 14 | 1061 | 1.034 | 0.341 | 3.031 | 2.813 (1.441–5.492) | 0.0024 |
| Hb AS | 159 | 916 | —0.673 | 0.223 | —3.017 | 0.510 (0.329–0.790) | 0.0026 |
| CSF2 AT haplotype | 485 | 590 | 0.291 | 0.122 | 2.396 | 1.338 (1.055–1.699) | 0.0166 |
| Model fit | AIC | 5509.852 | | | | | |
| Deviance | 2254.387 | 1063 | | | | |
| Null deviance | 2743.823 | 1074 | | | | |
| Model fit | AIC | 1360.7875 | | | | |
| Deviance | 861.3021 | 1068 | | | | |
| Null deviance | 972.6857 | 1074 | | | | |

Poisson regression model fit was determined using Akaike information criterion, with the heuristic approach being performed on an iterative manner to exclude potentially irrelevant variables. Data are ranked per variables as follows; the intercept, followed by metric variables (age), categorical variables (cohort, HIV1), genetic variables (sex, -α[3.7]/-α[3.7], Hb SS, Hb AS) and CSF2 gene variants. *Cohort presented in the table were patients recruited into the study in the 2007–2012 study period. IRR confidence intervals with GLM were computed using the delta method. Std. Error standard error, IRR incidence rate ratio, 95% CI 95% confidence interval, HIV 1 human immunodeficiency virus 1, -α[3.7]/-α[3.7] α-thalassemia homozygous mutant, Hb SS sickle cell diseases, Hb AS sickle cell trait, CSF2 colony-stimulating factor 2, AIC Akaike information criterion.
$\text{CSF2 AT haplotype, which increased the risk of SMA episodes ($P = 0.0166$) compared to the wild-type GT haplotype.}$

**Impact of CSF2 variants on the longitudinal risk of malaria infections**

After determining the impact of CSF2 variants on the rate of malaria and SMA episodes, we then determined the effect of the variants on the time between events using an ordered-events, generalized Cox proportional hazard model. Shown in Table 4 are the covariates that emerged from the model. Children who were older upon enrollment had a reduced hazard risk (HR) for malaria ($P<0.0001$). Although retained in the model, cohort ($P=0.1159$), HIV-1 positivity ($P=0.1711$), sex ($P=0.0580$) and $-\alpha^\alpha/-\alpha^\alpha$ ($P=0.0874$) were not significant predictors for the time between malaria infections (Table 4). However, inheritance of either the Hb SS mutant genotype ($P<0.0001$) or the Hb AS variant ($P=0.0001$) decreased the longitudinal hazard for malaria infections. Carriage of the CSF2 AC haplotype was also protective against malaria infections ($P=0.0045$) relative to wild-type haplotype (GT), while co-inheritance of the CSF2 GC/GT diplotype showed a non-significant trend towards a lower risk of malaria ($P=0.1108$) compared to wild type (GT/GT) (Table 4).

**Impact of CSF2 variants on the longitudinal risk of SMA infections**

Cox proportional hazard model was also used to examine the influence of CSF2 variants rs168681:G $>$ A and rs246835:G $>$ C on the time-to-event for malaria infections that culminated in the development of SMA (Table 4). Consistent with the Poisson models examining the rates of SMA, time-to-event modeling revealed that older children (at enrollment) had a lower risk of SMA ($P<0.0001$, i.e., the hazard decreases by a factor of 0.954 for each additional month of enrollment age) as did children enrolled in cohort 2 ($P = 0.0036$). Co-infection with malaria and HIV-1 was a strong predictor of increased susceptibility to SMA ($P < 0.0001$), as was carriage of the Hb SS genotype ($P = 0.0342$). Conversely, inheritance of the Hb AS variant decreased the longitudinal risk of SMA ($P = 0.0196$). Although non-significant, there was a trend towards increased risk of SMA in children with the CSF2 AT haplotype ($P = 0.0809$).

**Influence of CSF2 variants on the longitudinal risk of all-cause mortality**

The influence of covariates on the risk of all-cause mortality was investigated using a Cox proportional hazard model (Table 4). Older children (at enrollment) had a reduced risk of mortality ($P=0.0015$), whereas children with HIV-1 had a 16-times higher risk of mortality ($P<0.0001$). Children with Hb SS also had a marked increase in the risk of mortality ($P=0.0019$). Conversely, carriage of sickle cell trait (Hb AS) conferred a lower hazard risk for mortality ($P=0.0427$). Inheritance of the rs168681 GA genotype significantly increased the risk of dying during the follow-up period ($P=0.0315$), as did carriage of the CSF2 GC/GC diplotype, although not significant ($P=0.0592$).

**Discussion**

Advances in human gene mapping, along with an increased understanding of the molecular mechanisms of protective immunity, illustrate that susceptibility to malaria and its clinical outcomes is conditioned by genotypic variation [8]. The work presented here is focused on gaining an improved understanding of the molecular basis of clinical immunity to SMA in pediatric populations living under intense $P. falciparum$ transmission [8]. In such holoendemic regions, clinical immunity to malaria is mediated, at least in part, by the progressive generation of antibodies following repeated malaria infections [7, 24]. However, prior to the development of naturally acquired malarial immunity, children are reliant upon innate immune responses and can experience life-threatening complications that include severe anemia, hyperparasitemia, and respiratory distress [7, 8]. To better understand the pathogenesis of SMA, and as a consequence, reduce mortality, our studies primarily focus on genes and gene pathways involved in innate immunity. Consistent with this strategy, the current study investigated the influence of genetic variants flanking CSF2 (rs168681:G $>$ A and rs246835:T $>$ C) on the rate and timing of malaria and SMA over a 36-month longitudinal follow-up period during the developmental phase of naturally acquired malarial immunity. The impact of CSF2 gene variants on all-cause mortality was also investigated. Since other factors can influence the development of malaria, SMA, and mortality, we also determined the influence of important covariates in all statistical models.

Previous studies from our laboratories have shown that the host releases both pro- and anti-inflammatory cytokines, chemokines, growth factors, and effector molecules as part of the innate immune response to malaria infections [7, 8]. Among the growth factors is GM-CSF, which is highest among children with uncomplicated malaria relative to those with non-SMA and SMA [24]. However, children with SMA have higher levels of circulating GM-CSF than individuals with non-SMA, suggesting a complicated pattern of production during acute infection [24]. Studies by others have shown divergent results on clinical outcomes in malaria. For example, elevated GM-CSF levels are associated with severe malaria
complications (i.e., splenomegaly and leukocytosis) in some investigations, while others have found a protective role for GM-CSF [59]. Recent studies suggest that the production of TLR 7/8-driven GM-CSF in cord blood is an independent predictor of enhanced malaria risk over the first year of life, suggesting that GM-CSF indeed plays
an important role in malarial immunity [21]. To expand knowledge on the role of GM-CSF in malaria, we examined the impact of genetic variations around CSF2 on susceptibility to malaria and its severe disease manifestations (i.e., SMA and mortality) in children as they progressively develop immunity to clinical malaria.

Selection of the polymorphic variants was based on interrogating SNPs (and their combinations) that are previously unexplored in malaria and have the potential to impart functional changes on GM-CSF production. For example, the presence of the wild-type G allele at the rs168681:G>A locus within one distal enhancer produce TFBSs for ER-alpha and ZEB1, whereas transition to the A allele creates a binding site for NF-X3. Variation at rs246835:T>C locus within another distal enhancer of CSF2 has a TFBS for USF2 in the presence of the T allele and a TFBS for GATA-1 in carriers of the C allele. Previous studies have confirmed that CSF contains consensus elements for ZEB1 and GATA [27, 28]. Since transcription factors are adaptor molecules that detect regulatory sequences in the DNA and target the assembly of protein complexes that control gene expression, mutations within the transcription factor binding sites (TFBS) can alter gene expression [60]. The allele frequencies for the two SNPs in the study cohort were comparable to those in the Yoruba population (HapMap Project) and the African population (1000 Genome Project), suggesting that the two loci have been steadily maintained in ethnic groups of African descent [61]. The rs168681:G>A variant displayed HWE, whereas the rs246835:T>C locus had a significant departure from HWE in the overall population, and in each of the clinical groups investigated. Although a departure from HWE at rs246835:T>C locus could be attributed to historical pressure from malaria [62], there were comparable frequencies of the genotypes, as well as haplotypes and diplotypes in combination with rs168681:G>A across the clinical groups (i.e., aparasitemic, non-SMA, and SMA), suggesting an influence of a large sample size. This finding suggests that the two variants selected for investigation are likely not under strong selection pressure. The LD measures for the two loci with a $D'=0.416$ and $r^2=0.01$ indicate that the two variants are not strongly linked [63].

Analysis of the interacting covariates revealed that the children’s age, cohort (2003–2005 vs 2007–2012), sex (female vs male), and HIV-1 status (+ vs −) altered the incidences and risk of malaria and SMA over the longitudinal follow-up of 36 months. Further, co-inheritance of genetic variants; $α^+$-Thalassemia and/or sickle cell traits influenced longitudinal susceptibility to malaria and SMA infections and mortality outcomes. These results parallel those of previous studies that reported variability in the degree of protection conferred by hemoglobinopathies against mild and severe malaria (reviewed in [64]). In addition, the selected CSF2 homozygous and heterozygous variants (genotypes, haplotypes, and diplotypes) altered the incidence rate and hazard ratios to malaria and SMA over the follow-up period. Results presented here complement previous studies illustrating that children living in regions with high transmission rates for P. falciparum experience multiple infections prior to developing malarial immunity, with the greatest burden of severe disease manifesting in children less than 5 years of age [8, 50–52]. As such, longitudinal studies have a greater ability to detect genetic factors, and covariates that can influence both the rate and timing of repeated malaria episodes and the development of severe disease over time.

A Poisson regression model estimating the rate of malaria episodes over 36 months revealed that the presence of the rs246835 TC heterozygote genotype and CSF2 AC/GC diplotype decreased the incidence of malaria, while the CSF2 AT/GC diplotype increased the incidence of malaria episodes. Additional Poisson analysis identified significantly more episodes of SMA among children who inherited the CSF2 AT haplotype. To further elucidate the impact of the CSF2 genetic variants on the longitudinal risk of malaria and SMA, a Cox proportional hazard model was fit with independent increments according to the Anderson–Gill method. Carriage of the CSF2 AC haplotype was associated with a lower hazard risk for malaria infections. Additionally, inheritance of the CSF2 AT haplotype that significantly increased the incidence rate for SMA was associated with an elevated longitudinal hazard for SMA, albeit non-significant. Results obtained in this study support our previous findings that showed innate immune response genes influence longitudinal susceptibility to malaria and subsequent development of SMA in this P. falciparum holoendemic region [50–52]. Moreover, results presented here illustrate that CSF2 genetic variants are associated with both the rate and timing of malaria and SMA episodes during the development of naturally acquired malarial immunity in children living in a high malaria transmission region. Although transcript and protein levels in circulation for CSF2/GM-CSF were not measured in the current study due to sample availability, we hypothesize that the variants alter the gene products and, thereby, influence susceptibility to malaria and SMA, as demonstrated in our previous studies for other genetic variants [50, 61].

We also investigated predictors of all-cause mortality across 36 months using Cox proportional hazard modeling and found that younger children and those with HIV-1 had an increased risk of death. In addition, carriage of the Hb AS trait protected against mortality,
whereas inheritance of the Hb SS homozygous mutant imparted 6.5 times higher risk of dying. Several CSF2 variants were also associated with increased susceptibility to all-cause mortality, including carriage of the rs168681 GA genotype (1.8 times higher) and the CSF2 GC/GC diplotype (2.6 times higher). Interestingly, neither of these variants significantly impacted susceptibility to either malaria or SMA, suggesting that their impact on mortality may be due to non-malaria-related causes.

Conclusions
In summary, although the two variants investigated correspond to distal enhancer regions within the 5q31.1, and are associated with altered susceptibility to malaria, SMA, and all-cause mortality, the underlying molecular mechanism(s) for the disease associations remain largely unknown. To our knowledge, this is the first study examining the impact of two genetic variants flanking the 5q31.1 gene region on longitudinal malaria disease outcomes and all-cause mortality. It will be important for future studies to establish the precise role of CSF2, and the soluble inflammatory mediator it encodes, GM-CSF, on susceptibility to malaria, and the subsequent development of severe disease once an individual becomes infected.

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Author contributions
LEK participated in SNP selection, generated genotyping and GM-CSF levels determination, data analyses, and manuscript writing. ER, EOM, and CO: project supervision, and manuscript review and editing. QC, KC, and PK participated in SNP selection technical support, and manuscript review and editing. BHM, NWH, NL, CGL and KAS performed longitudinal data analysis, statistical interpretations, wrote portions of R code, and manuscript review and editing. DJP designed clinical and experimental studies, data analyses, manuscript writing and editing, provided clinical samples and data, and supplied reagents, materials, and analyses tools. SBA provided technical support, performed longitudinal data analyses, statistical interpretations, manuscript writing and editing. All authors read and approved the final manuscript.

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Availability of data and materials
All data generated or analyzed during this study are included in this published article (and its additional information files).

Declarations
Ethics approval and consent to participate
The study was approved by the ethics and scientific review committee of the Kenya Medical Research Institute (KEMRI), Maseno University Ethics Review Committee (MUERC), and University of New Mexico Institutional Review Board. Parents/legal guardians of children participating in the study provided written informed consent. All research was performed according to stipulated guidelines and regulations of the approving and participating institutions.

Consent for publication
Parents/legal guardians of children participating in the study provided consent for publication.

Competing interests
The authors declare that they have no competing interests.

Author details
1 Department of Biomedical Sciences and Technology, School of Public Health and Community Development, Maseno University, Maseno, Kenya. 2 University of New Mexico-Kenya Global Health Programs, Kismu, Siaya, Kenya. 3 Center for Global Health, University of New Mexico, Albuquerque, NM, USA. 4 Department of Medical Laboratory Sciences, School of Biomedical Sciences and Technology, Masinde Muliro University of Science and Technology, Kakamega, Kenya. 5 Department of Clinical Medicine, School of Health Sciences, Kirinyaga University, Kerugoya, Kenya. 6 Theoretical Biology and Biophysics Group, Theoretical Division, Los Alamos National Laboratory, Los Alamos, NM, USA. 7 Center for Global Health Research, Kenya Medical Research Institute, Kismu, Kenya. 8 Department of Medicine, Loyola University Medical Center, Chicago, IL, USA. 9 Department Applied Computer and Bio-Sciences, University of Applied Sciences Mittweida, Mittweida, Germany. 10 Department of Medical Biochemistry, School of Medicine, Maseno University, PO. Box 333-40105, Maseno, Kenya.

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