

NOS2 Variants Reveal a Dual Genetic Control of Nitric Oxide Levels, Susceptibility to *Plasmodium* Infection, and Cerebral Malaria

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Nitric oxide (NO) is a proposed component of malaria pathogenesis, and the inducible nitric oxide synthase gene (*NOS2*) has been associated to malaria susceptibility. We analyzed the role of *NOS2* polymorphisms on NO bioavailability and on susceptibility to infection, *Plasmodium* carrier status and clinical malaria. Two distinct West African sample collections were studied: a population-based collection of 1,168 apparently healthy individuals from the Príncipe Island and a hospital-based cohort of 269 Angolan children. We found that two *NOS2* promoter single-nucleotide polymorphism (SNP) alleles associated to low NO plasma levels in noninfected individuals were also associated to reduced risk of pre-erythrocytic infection as measured anti-CSP antibody levels ($6.25E-04 < P < 7.57E-04$). In contrast, three SNP alleles within the *NOS2* cistronic region conferring increased NO plasma levels in asymptomatic carriers were strongly associated to risk of parasite carriage ($8.00E-05 < P < 7.90E-04$). Notwithstanding, three SNP alleles in this region protected from cerebral malaria ($7.90E-4 < P < 4.33E-02$). Cohesively, the results revealed a dual regimen in the genetic control of NO bioavailability afforded by *NOS2* depending on the infection status. *NOS2* promoter variants operate in noninfected individuals to decrease both NO bioavailability and susceptibility to pre-erythrocytic infection. Conversely, *NOS2* cistronic variants (namely, rs6505469) operate in infected individuals to increase NO bioavailability and confer increased susceptibility to unapparent infection but protect from cerebral malaria. These findings corroborate the hypothesis that NO anti-inflammatory properties impact on different steps of malaria pathogenesis, explicitly by favoring infection susceptibility and deterring severe malaria syndromes.

Malaria is the result of a multistage *Plasmodium* infection that elicits a multiplicity of host responses. Inflammatory responses are determinants of the clinical course of infection and are influenced by host genetic factors (1). Genetic evidence accumulated in recent years supports a complex role for host genetics in resistance and susceptibility to human malaria (2). Hemoglobin gene variants are well-known malaria resistance factors, but a considerable number of genetic studies focused on clinical malaria syndromes and blood parasite burden also highlighted genes involved in the immune response, inflammation, and cell adhesion (1).

Nevertheless, the exact role of genetic variance in inflammatory responses against *Plasmodium* infection and in malaria severity remains unclear (1). It is possible that innate immunity genes associated to malaria may play a dual role in the course of infection. Proinflammatory factors would favor an efficacious anti-parasite response leading to parasite clearance and therefore conferring a lower degree of susceptibility to unapparent and mild infections. On the other hand, such factors could increase the risk of developing strong inflammatory responses that trigger severe inflammatory syndromes, namely, cerebral malaria.

Nitric oxide (NO) has been proposed to play a relevant role in malaria pathogenesis, but its mechanisms of action in different stages of infection remain to be elucidated (3). The *NOS2* gene codes for the inducible nitric oxide synthase (iNOS) that is responsible for high-level production of NO by activated phagocytes (4). Several studies focused on *NOS2* promoter polymorphisms have reported genetic association to different malaria clinical out-

comes (5–12), but the role of such variants in malaria infection progression and nitric oxide production appears to be complex (13). Moreover, it is unclear whether *NOS2* genetic variants play a role in susceptibility to asymptomatic malaria (14, 15).

Asymptomatic malaria infections have been frequently described in regions where malaria is endemic in both high- and intermediate-transmission areas (16–23). Asymptomatic malaria is suggested to represent an immunological state developed upon repeated exposure that tolerates the parasite in the absence of clinical symptoms (clinical immunity). On the other hand, such unapparent infections are an implicit manifestation of premunition, an immune response that enables control of blood parasite burden at low levels but do not efficiently lead to complete elimination of *Plasmodium* parasites (24). The mechanisms involved in the acquisition of premunition responses in exposed individu-

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als remain elusive, but some reports have suggested that protection against asymptomatic *Plasmodium* infection (25) and the malaria reservoir status (23, 26) are influenced by host genetic factors.

To study the involvement of *NOS2* gene in controlling NO bioavailability, malaria susceptibility, and severe disease, we analyzed a population-based collection of apparently healthy individuals, conducted in 2005 in the Príncipe Island on the West Coast of Africa and a hospital-based collection of Angolan children with uncomplicated and cerebral malaria. Using markers of current and past infection in apparently healthy individuals of the Príncipe collection, we analyzed the effect of *NOS2* gene variants in susceptibility to acquire infection and their role in controlling NO plasma levels in infected and noninfected individuals. Furthermore, in clinical malaria samples we analyzed the role of *NOS2* gene variants in susceptibility to cerebral malaria (CM). We report that *Plasmodium* infection impacts on the control of NO bioavailability by *NOS2* genetic variants and that distinct *NOS2* gene regions are associated with infection susceptibility and with the risk of clinical malaria progression.

MATERIALS AND METHODS

Ethics. Ethical permit to conduct the present study in the Príncipe collection was granted by the Ministry of Health of São Tomé and Príncipe in the scope of a collaborative protocol on malaria research between the Fundação Calouste Gulbenkian (Portugal) and the government of São Tomé and Príncipe. Ethical permit for the study in the Angola collection was granted by the Ethical Committee of the Hospital Pediátrico David Bernardino (HPDB) in Luanda, appointed by the Angolan Ministry of Health. All investigations were conducted according to the principles expressed in the Declaration of Helsinki. Informed consent was obtained from every participant and, in case of children, consent was obtained from their guardians.

Sample collection. The Island of Príncipe covers ~55 square miles (142 square kilometers) with about 6,000 inhabitants representing a well-circumscribed, nonmigratory community. The Príncipe collection resulted from a cross-sectional study conducted in 2005 that covered 10 villages in the Northern Príncipe Island (within a 50-km² region) and enrolled 1,387 apparently healthy donors within a period of 3 weeks. Blood samples were collected from participants without any prior selection or restrictions on participation. The age distribution of the sampled individuals represented the whole population, ranging from 1 month to 85 years of age. Individuals with tracked relatedness up to the second ancestor generation to a participant were excluded from the study.

The details of the Angola collection have been described previously (27). Patients were selected from attendees to the HPDB from February 2005 to May 2007. Malaria was diagnosed on the basis of a positive asexual parasitemia detected on a Giemsa-stained thick smear (28). CM was defined according to the World Health Organization criteria: either a coma score of <3 on the Blantyre scale for children <60 months or a coma score <7 on the Glasgow scale for children ≥60 months. Meningitis and encephalitis were ruled out by cerebrospinal fluid analysis after lumbar puncture. Cases with known causes of encephalopathy and hypoglycemia (glycemia < 40 mg/dl) were excluded. The UM group represents patients with malaria diagnosis by microscopic examination and febrile illness without any clinical finding suggestive of other causes of infection and with no manifestations of severe malaria.

Plasmodium detection. Parasite DNA was extracted from frozen packed red blood cells using a 96 QIAmp blood kit (Qiagen, USA). A nested PCR amplification assay was used for the detection of the main *Plasmodium* species of human malaria (*P. falciparum*, *P. vivax*, *P. malariae*, and *P. ovale*) as described previously (29). In the Príncipe collection, asymptomatic individuals were considered *Plasmodium* carriers if

they tested positive for any *Plasmodium* species. In the Angola samples, malaria laboratory diagnosis was confirmed using this method.

ELISA. In the Príncipe collection, IgG antibodies against *P. falciparum* circumsporozoite protein (CSP) were detected by enzyme-linked immunosorbent assay (ELISA) in the 1,209 plasma samples using PpCSP-M2 antigen (30). Microtiter plates were coated with antigen (5 µg/ml) in coating buffer was added, followed by incubation overnight at 4°C. The antigen solution was then removed, and the plate was washed three times with phosphate-buffered saline (PBS). After blocking with PBS-gelatin (1%), a 100-µl aliquot of 1/100 diluted plasma in PBS-gelatin-Tween was incubated overnight at 4°C, followed by five washes with PBS. As a secondary antibody, 100 µl of 1/600 dilution of goat anti-human IgG coupled to alkaline phosphatase (Sigma-Aldrich, USA) in PBS-Gelatin-Tween was incubated for 2 h at 37°C. The supernatant was discarded and the plates were washed five times with PBS and incubated with 100 µl of substrate solution (10 mg of pNPP in 1 ml of 1 M Tris [pH 9.8] plus 9 ml of 1.5 M NaCl). Known positive and negative control plasma samples were included in each plate. The absorbance at 405 nm was determined by using a micro-ELISA plate reader, and results were expressed in arbitrary units (AU) calculated as follows: $\log [(A_{405}\text{-test plasma} - A_{405}\text{-blank}) / (A_{405}\text{-positive control} - A_{405}\text{-blank}) \times 10,000]$. ELISA specificity was evaluated in 44 samples of unexposed European individuals that showed very low or undetectable levels of anti-CSP antibodies. To ensure specificity, the positivity cutoff value (>0.90 AU) was set at the AU average + 4× standard deviation obtained in 44 samples of unexposed European individuals.

Nitric oxide quantification. Plasma NO levels (µM) were assessed by chemiluminescence-based measurement of nitrate (NO₃⁻) and nitrite (NO₂⁻) concentrations, as previously described (31). This method is based on the vanadium III-induced reduction of NO₂⁻ and NO₃⁻ to NO, at a high temperature (90°C), using a Sievers 280 NO analyzer (Sievers Instruments, USA).

Genotyping. Genomic DNA was extracted from whole blood using Chemagen magnetic beads technology. DNA preparations were quantified using PicoGreen reagents (Invitrogen, Portugal) according to the supplier's instructions. A total of 25 single-nucleotide polymorphisms (SNPs) covering the *NOS2* region were initially genotyped by using the Sequenom iPLEX assay (Sequenom, San Diego, CA) and the Sequenom MassArray K2 platform at the Genomics Unit of the Instituto Gulbenkian de Ciência. Extensive quality control was performed using eight HapMap (<http://hapmap.ncbi.nlm.nih.gov/>) controls of diverse ethnicity, a Hardy-Weinberg equilibrium (HWE) with $P > 0.01$, and a minimum of 90% call rate for each SNP. Genotype determinations were performed blinded to affection status. One SNP (rs2297514) was excluded due to a genotyping failure. Furthermore, four SNPs did not meet the quality control criteria—three in both sample populations (rs28973255, rs9895831, and rs9906835) and one (rs28998790) in the Angola collection—and were also excluded. Samples with <75% call rate and duplicates were excluded from analysis, such that 45 samples from Príncipe and 3 from Angola were removed from the analysis.

Genetic analysis. The final data set used in the analysis consisted of 1,168 subjects that had both genetic and plasma data for the Príncipe population and 269 individuals for the Angolan collection comprising 127 CM children and 142 UM patients. Association analysis of asymptomatic carrier status (*Plasmodium* PCR-positive individuals) in the Príncipe population and cerebral malaria in the Angolan population was performed by logistic regression (adjusted for age and gender) using the SNPAssoc v1.4-9 package (32) implemented in the R freeware (<http://cran.r-project.org/>). The results were considered suggestive below a conventional level of 0.05. Bonferroni corrections for multiple tests were carried out to exclude type I errors (the significance levels for 21 tests in the Príncipe population and 20 tests in the Angolan population are set at $P < 2.38 \times 10^{-3}$ and at $P < 2.50 \times 10^{-3}$, respectively). χ^2 tests for HWE in the sample populations, allelic and haplotypic association, and linkage disequilibrium (LD) analysis were performed using Haploview 4.2 (33). Quantita-

TABLE 1 Prevalence of infection markers in apparently healthy individuals

Collection	Total no. of subjects	Age range in yrs (median)	No. male	No. female	OR (95% CI); Covariance <i>P</i> value ^a	
					Age	Gender
PCR ⁺	378	0–75 (13.5)	174	204	0.99 (0.98–0.99); 0.045	0.85 (0.67–1.09); NS
PCR [–]	790	0–85 (16.0)	332	457	0.99 (0.98–0.99); 0.045	0.85 (0.67–1.09); NS
CSP ⁺	973	0–85 (17.0)	419	553	1.02 (1.00–1.02); 8.0E–04	1.08 (0.79–1.49); NS
CSP [–]	195	0–74 (8.0)	87	108	1.02 (1.00–1.02); 8.0E–04	1.08 (0.79–1.49); NS

^a Logistic regression odds ratios (OR) and 95% confidence intervals (CI) are indicated. NS, not significant.

tive trait locus (QTL) analysis was performed to test the main effect of each tested *NOS2* SNP on anti-CSP antibody titers (CSP QTL) and NO concentration (NO QTL) in the Príncipe population. At each SNP, anti-CSP antibody titers and the NO concentration were regressed onto genotype counts in a regression model that included gender and age as covariates. The SNPassoc v1.4-9 package (32) implemented in the R freeware was used for logistic regression analysis. Results with a *P* value of <0.05 were considered evidence for suggestive association.

RESULTS

Infection markers in apparently healthy individuals. The sample collection of apparently healthy individuals was conducted in the Príncipe Island population in May 2005 when the disease transmission for malaria coursed at the mesoendemic level. Based on the criteria set in Materials and Methods, the final data set analyzed in the present study consisted of 1,168 subjects that had both genetic and phenotype data. *Plasmodium* carriage in apparently healthy individuals was evaluated by determining subpatent parasitemia scored by PCR, and susceptibility to infection was indirectly ascertained by quantifying antibodies against CSP that represent an immunological marker of repeated pre-erythrocytic infection (34). We found that 32.3% of the apparently healthy population carried *Plasmodium* DNA in peripheral blood (malaria asymptomatic carriers), while 79.8% of the population was positive for antibodies against CSP (Table 1 and Fig. 1). The asymptomatic carrier status was marginally dependent on age but, as expected, anti-CSP positivity was significantly influenced by age (odds ratio [OR] = 1.02, *P* = 8.00E–04), presumably reflecting the natural history to parasite exposure (Table 1). Nevertheless, parasite carriage was not dependent on declared ethnic ancestry or on demography, indicating that local epidemiological variables in Príncipe did not significantly influence the asymptomatic malaria status (see Table S1 in the supplemental material). Not surprisingly, CSP antibody titers were higher in parasite

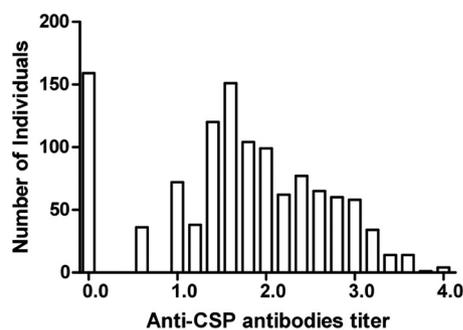


FIG 1 Distribution of plasma IgG antibody levels against *Plasmodium* circumsporozoite protein (CSP) in apparently healthy individuals. Antibody levels are presented in arbitrary units, and the positivity cutoff value is 0.90, as described in Materials and Methods.

carriers, but the proportion of individuals negative for CSP antibodies among asymptomatic carriers (14%) suggested that asymptomatic infection did not prevent the detection of individuals lacking antibody markers of repeated pre-erythrocytic infection (see Fig. S1 in the supplemental material). These results suggested that these two infection markers represent different criteria in ascertaining malaria susceptibility.

In this mesoendemic setting, a significant proportion of the apparently healthy population carried parasitological markers of current infection. Nevertheless, a sizable fraction of the population with lifetime exposure does not show antibody markers of previous pre-erythrocytic infection. This suggested that host factors were influencing susceptibility to asymptomatic carrier status and persistent susceptibility to infection.

***NOS2* variants are associated to the asymptomatic carrier status.** To test whether *NOS2* gene variants were involved in malaria asymptomatic carrier status, we genotyped 1,168 individuals of the Príncipe collection for 21 *NOS2* SNPs that spanned the promoter and the cistronic gene region (Fig. 2). We compared genotype frequencies in asymptomatic individuals against the general population. The age- and gender-corrected analysis under the genetic log-additive model indicated that three intronic SNP alleles (SNP 10/allele A, SNP 12/allele T, and SNP 15/allele T) were associated with susceptibility to malaria asymptomatic carrier status, with the highest association occurring at rs3794767 (*P* = 8.00E–05; OR = 1.53; 95% confidence interval [95% CI] = 1.24 to 1.88) (Fig. 2B and Table 2). In contrast, two other SNP alleles in the promoter region of *NOS2* (SNP 20/allele A and SNP 21/allele A) were associated with decreased asymptomatic carrier susceptibility (*P* = 4.18E–2 and *P* = 1.51E–2, respectively), as denoted by OR < 1 (Fig. 2B and Table 2). Three of the five associated markers remained significantly associated after conservative Bonferroni correction for multiple testing (*P* ≤ 2.38E–3) (Table 2). The three intronic SNPs were in strong linkage disequilibrium (LD; *r*² ≥ 0.73) and together with four other SNPs in this region compose a LD block (block 2 in Fig. 2D), which was separate from the LD block that included the two SNPs in the promoter region (block 3). Haplotypic association analysis comprising the SNPs in each of the three LD blocks (Fig. 2) did not identify haplotypes with a stronger association than the individual SNPs (data not shown). These results suggest that the major *NOS2* effect implicated in increasing the risk of parasite carriage was mapping within the LD block that contains rs3794767.

***NOS2* variants are associated to susceptibility to pre-erythrocytic infection.** Next, we evaluated whether markers of pre-erythrocytic infection corroborated *NOS2* association with asymptomatic malaria. QTL analysis of anti-CSP antibody plasma levels was performed in the Príncipe sample (1,168 individuals). Interestingly, we found that the three intronic SNP alleles (SNP

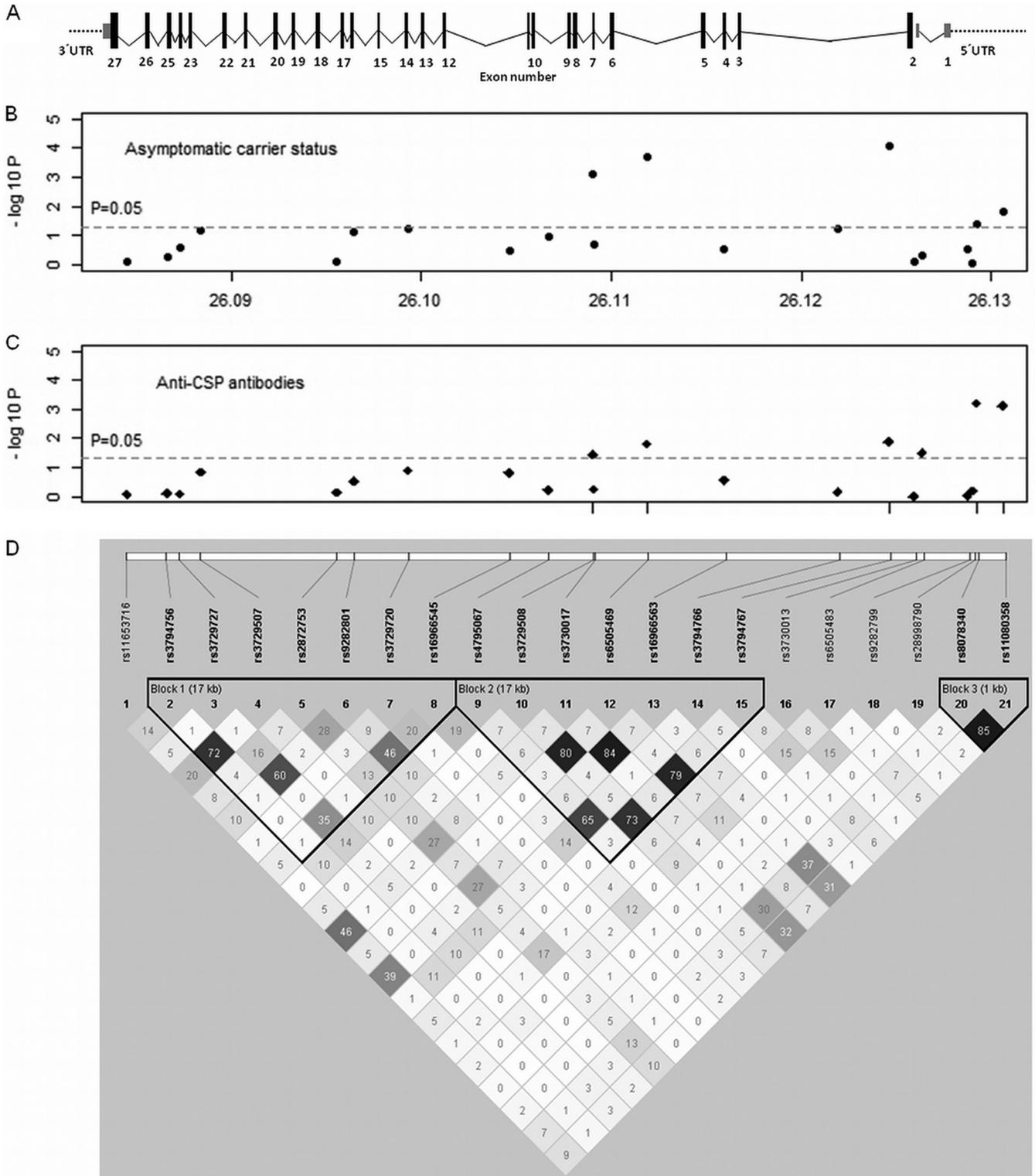


FIG 2 Genetic association of *NOS2* SNPs with asymptomatic malaria and anti-CSP antibodies in the Principe population. (A) *NOS2* gene structure scaled diagram. Exons are represented by boxes and numbered. (B and C) Plots of association tests for the healthy carrier status under the log-additive model (B) and QTL analysis of anti-CSP antibody levels (C) for the represented SNPs. The results are presented as the $-\log_{10}$ of the *P* value. (D) LD map for the tested SNPs obtained using Haploview 4.2 (values represent pairwise r^2).

TABLE 2 Genotypic association of NOS2 SNPs to asymptomatic malaria^a

SNP no.	SNP reference	Position (Mb)	Gene region ^b	Alleles ^c	Minor allele frequency	Genotypes	Asymptomatic carriers (n = 378)		P _{adj}	OR (95% CI)	P _{corr}
							Noncarriers (n = 790)				
1	rs11653716	26084532	Intron 26	G/C	0.274	CC/GC/GG	190/156/24	407/318/53	0.7856	0.97 (0.58–1.63)	NS ^d
2	rs3794756	26086629	Intron 25	T/C	0.047	CC/TC/TT	304/34/0	653/57/4	0.5389	1.14 (0.75–1.74)	NS
3	rs3729727	26087316	Intron 24	A/G	0.172	GG/AG/AA	272/90/15	531/233/24	0.2751	0.88 (0.70–1.11)	NS
4	rs3729507	26088432	Intron 22	C/G	0.079	GG/CG/CC	308/66/2	681/96/9	0.0674	1.34 (0.98–1.81)	NS
5	rs2872753	26095562	Intron 17	G/A	0.460	AA/GA/GG	102/201/75	219/418/53	0.7638	1.03 (0.86–1.23)	NS
6	rs9282801	26096473	Intron 16*	T/G	0.248	GG/TG/TT	235/116/27	430/309/50	0.0784	0.83 (0.68–1.02)	NS
7	rs3729720	26099324	Intron 14*	T/C	0.038	CC/TC/TT	357/19/1	723/66/1	0.0568	0.63 (0.38–1.03)	NS
8	rs16966545	26104622	Intron 11	A/T	0.165	TT/AT/AA	273/92/12	535/233/17	0.3173	0.89 (0.70–1.13)	NS
9	rs4795067	26106675	Intron 9	G/A	0.195	AA/GA/GG	261/102/15	505/245/39	0.1082	0.84 (0.67–1.04)	NS
10	rs3729508	26109030	Intron 7*	A/G	0.230	GG/AG/AA	191/152/25	489/253/36	7.90E–04	1.42 (1.16–1.75)	1.66E–02
11	rs3730017	26109102	Exon 7	T/C	0.210	CC/TC/TT	242/123/12	481/274/35	0.2054	0.87 (0.70–1.08)	NS
12	rs6505469	26111886	Intron 5	T/A	0.231	AA/TA/TT	189/152/27	490/259/33	1.90E–04	1.48 (1.21–1.82)	3.99E–03
13	rs16966563	26115949	Exon 4	C/T	0.214	TT/CT/CC	240/124/14	483/263/42	0.2849	0.89 (0.72–1.10)	NS
14	rs3794766	26121921	Intron 2	T/C	0.173	CC/TC/TT	275/81/15	524/228/30	0.0569	0.80 (0.63–1.01)	NS
15	rs3794767	26124605	Intron 2	T/C	0.211	CC/TC/TT	198/146/23	515/231/30	8.00E–05	1.53 (1.24–1.88)	1.68E–03
16	rs3730013	26125918	Intron 1*	T/C	0.247	CC/TC/TT	211/151/16	434/311/40	0.7463	0.97 (0.78–1.19)	NS
17	rs6505483	26126345	Intron 1	A/G	0.229	GG/AG/AA	217/139/20	473/273/40	0.4777	1.08 (0.88–1.33)	NS
18	rs9282799	26128728	Promoter	T/C	0.047	CC/TC/TT	333/38/1	712/68/0	0.2877	1.25 (0.83–1.88)	NS
19	rs28998790	26129014	Promoter	A/G	0.066	GG/AG/AA	327/46/2	684/98/3	0.9266	1.02 (0.72–1.44)	NS
20	rs8078340	26129212	Promoter	A/G	0.279	GG/AG/AA	213/130/28	390/317/68	4.18E–02	0.82 (0.67–0.99)	NS
21	rs11080358	26130596	Promoter	A/G	0.250	GG/AG/AA	226/128/17	421/309/53	1.51E–02	0.77 (0.63–0.95)	NS

^a Association tests were performed using the log-additive model, and the results were adjusted for age and gender (P_{adj}). Significant adjusted P values (P_{adj} < 0.05) and significant Bonferroni-corrected P value (P_{corr} < 0.05) are highlighted in boldface.

^b *, rs9282801 is located in intron 16 in a boundary region to exon 16 (at 88 bp), rs3729720 is located in intron 14 in a boundary region to exon 14 (at 10 bp), rs3729508 is located in intron 7 in a boundary region to exon 7 (at 11 bp), and rs3730013 is located in intron 1 in a boundary region to exon 2 (at 10 bp).

^c Minor/major frequency alleles are indicated in this column.

^d NS, not significant.

10/allele A, SNP 12/allele T, and SNP 15/allele T) that appeared to increase the risk of asymptomatic malaria were also significantly associated with elevated levels of anti-CSP antibodies (Fig. 2C and Table 3). Conversely, we found that the two SNP alleles in the promoter region (SNP 20/allele A and SNP 21/allele A) that were protecting subjects from parasite carrier status were strongly associated with low levels of anti-CSP antibodies (Table 3). Since anti-CSP levels were higher in asymptomatic carriers (see Fig. S1 in the supplemental material), we performed the same analysis, correcting for the malaria carrier status. Interestingly, only the association signals in promoter SNPs remained significant (Table 3), suggesting that the promoter region is a major factor in controlling susceptibility to pre-erythrocytic infection, as ascertained from the anti-CSP antibody levels. These results revealed that NOS2 variants exert a dual genetic control in susceptibility to malaria. (i) The promoter alleles are overrepresented in noncarriers

and in individuals with low levels of anti-CSP antibodies, implying an association to reduced susceptibility to infection. (ii) On the other hand, the overrepresentation of intronic alleles in carriers and in individuals with higher levels of anti-CSP antibodies suggests their association with increased susceptibility to infection.

NOS2 variants associated to Plasmodium infection susceptibility control NO plasma levels. We sought to test whether the five NOS2 SNPs associated with malaria susceptibility were also controlling NOS2 activity. NO plasma levels were measured in a random subset of participants, 132 of which were noncarriers (mean ± the standard deviation [SD] = 51.9 ± 28.4 μM) and 84 of which were asymptomatic carriers (NO mean ± SD = 63.3 ± 40.5 μM). This difference in NO plasma levels (P = 2.30E–02) raised the possibility that NOS2 regulation was conditioned by malaria infection and justified a separate analysis of asymptomatic

TABLE 3 QTL analysis of NOS2 SNPs that control anti-CSP antibody levels in the Principe population^a

SNP no.	SNP reference	Gene region	Genotype	Genotype nb (n = 1168)		Mean antibody level	Mean difference (95% CI)	P _{adj}	Mean difference (95% CI)	P _{adj}
				1168	Mean antibody level					
10	rs3729508	Intron 7*	GG/AG/AA	680/405/61	1.645/1.764/1.780	0.098 (0.007–0.189)	3.51E–02	0.033 (–0.050–0.116)	0.4412	
12	rs6505469	Intron 5	AA/TA/TT	679/411/60	1.635/1.768/1.817	0.112 (0.022–0.203)	1.55E–02	0.040 (–0.043–0.124)	0.3445	
15	rs3794767	Intron 2	CC/TC/TT	713/377/53	1.634/1.791/1.755	0.118 (0.025–0.212)	1.33E–02	0.040 (–0.046–0.126)	0.3622	
20	rs8078340	Promoter	GG/AG/AA	603/447/96	1.760/1.661/1.433	–0.146 (–0.23–0.063)	6.25E–04	–0.111 (–0.187–0.034)	4.56E–03	
21	rs11080358	Promoter	GG/AG/AA	647/437/70	1.757/1.656/1.378	–0.151 (–0.238–0.063)	7.57E–04	–0.106 (–0.186–0.026)	9.34E–03	

^a Analysis was performed using the log-additive model, and the results were adjusted for age and gender (first P_{adj} column) and for age, gender, and asymptomatic status (second P_{adj} column). Significant P values (P_{adj} < 0.05) are highlighted in boldface. Mean difference refers to the mean antibody level difference. Introns denoted by asterisks are discussed in Table 2, footnote b.

TABLE 4 QTL analysis of NO plasma levels control by NOS2 SNPs in noncarriers^a

SNP no.	SNP reference	Gene region	Alleles ^b	Genotypes	Genotype nb (<i>n</i> = 132)	Mean NO level	Mean difference (95% CI)	<i>P</i> _{adj}
10	rs3729508	Intron 7*	A/G	GG/AG/AA	76/47/5	48.55/59.41/41.79	5.22 (-3.62–14.05)	0.2498
12	rs6505469	Intron 5	T/A	AA/TA/TT	71/52/7	47.92/59.36/36.98	3.49 (-4.90–11.87)	0.4163
15	rs3794767	Intron 2	T/C	CC/TC/TT	76/46/6	49.32/59.25/36.12	2.39 (-6.25–11.02)	0.5890
20	rs8078340	Promoter	A/G	GG/AG/AA	71/48/9	55.65/48.97/34.01	-8.61 (-16.55–0.66)	3.57E-02
21	rs11080358	Promoter	A/G	GG/AG/AA	74/48/7	56.16/47.19/38.64	-8.67 (-16.93–0.41)	4.17E-02

^a Analysis was performed using the log-additive model, and the results were adjusted for age and gender (*P*_{adj}). Significant *P* values are highlighted in boldface. Introns denoted by asterisks are discussed in Table 2, footnote b.

^b Indicated as minor/major frequency alleles.

and noncarrier individuals. QTL analysis revealed that the two SNP alleles in the promoter region (SNP 20/allele A and SNP 21/allele A) conferring a reduced risk of parasite carriage and pre-erythrocytic infection were also associated with lower levels of plasma NO in noncarriers (Table 4). In contrast, the three intronic SNP alleles (SNP 10/allele A, SNP 12/allele T, and SNP 15/allele C) associated with an increased risk of *Plasmodium* carriage and pre-erythrocytic infection also conferred higher plasma NO levels among asymptomatic carriers (Table 5).

These findings strongly suggest that the major genetic determinants of NO production controlled by NOS2 are distinct in parasite carriers and noncarriers. These results also show that the effects of promoter and intronic NOS2 SNP alleles on the susceptibility to *Plasmodium* infection paralleled their effects on NO plasma levels. Together, the data indicate that increased NO bioavailability correlates with higher susceptibility to parasite carriage and pre-erythrocytic infection, supporting the notion that NO plays an anti-inflammatory role in the context of malaria infection and decreases the efficacy of antiparasite responses.

NOS2 variants governing NO production are associated with clinical malaria progression. We used hospital-based samples to investigate whether NOS2 SNP variants control progression of clinical malaria manifestations, namely, from uncomplicated malaria to cerebral malaria. We compared the genotype frequencies of 20 NOS2 SNPs in a sample collection of 269 Angolan children (see Table S2 in the supplemental material) that presented with uncomplicated malaria (*n* = 142) or developed cerebral malaria (*n* = 127).

We found that three SNP alleles (SNP 11/allele T, SNP 12/allele T, and SNP 13/allele C) conferred protection against cerebral malaria, as denoted by an OR of <1 (Table 6). This association signal overlapped the gene region that confers susceptibility to infection (Tables 2 and 3) and increased NO levels in asymptomatic carriers (Table 5). Specifically, SNP 12 (rs6505469) allele T was associated with increased NO levels and susceptibility to infection in Príncipe but protected against cerebral malaria in Angolan children. On the

other hand, the SNP alleles in the promoter region did not show an association with clinical malaria.

The Angolan population showed a LD structure similar to the Principe population (see Fig. S2 in the supplementary material and Fig. 2, respectively). Specifically, the three intronic SNPs (SNPs 10, 12, and 15) in strong LD ($r^2 \geq 0.67$), together with SNPs (SNPs 9, 11, 13, and 14) composed comparable LD blocks. Likewise, the two SNPs in the promoter region showed strong LD ($r^2 = 0.79$). Haplotypic analysis of association with progression to cerebral malaria, comprising the SNPs in each of the three LD blocks, did not identify haplotypes with a higher significant association than the individual SNPs. We thus identified here two distinct regions in the NOS2 gene that control susceptibility to malaria in different stages of infection and demonstrated that the control of NO plasma levels by these two gene regions is dependent on the infection status.

DISCUSSION

We analyzed the role of NOS2 polymorphisms in governing NO plasma levels and the risk of *Plasmodium* carriage, susceptibility to pre-erythrocytic infection, and cerebral malaria. We found that two NOS2 SNP alleles in the promoter region that were associated with decreased NO plasma levels in noncarriers also conferred decreased susceptibility to pre-erythrocytic infection, as ascertained from the anti-CSP antibody levels. This suggests that decreased NO bioavailability controlled by the NOS2 promoter acts to decrease susceptibility to *Plasmodium* productive infection. Conversely, we identified a gene region associated with high NO plasma levels in asymptomatic carriers that conferred susceptibility to *Plasmodium* carriage and pre-erythrocytic infection but afforded protection against cerebral malaria. This implies that a distinct NOS2 gene region contributing to the control of NO bioavailability acts in infected individuals and impacts infection progression and disease severity.

Several genetic studies on the association of NOS2 polymorphisms with malaria in different populations have focused on ge-

TABLE 5 QTL analysis of NO plasma levels control by NOS2 SNPs in asymptomatic carriers^a

SNP no.	SNP reference	Gene region	Alleles ^b	Genotypes	Genotype nb (<i>n</i> = 84)	Mean NO level	Mean difference (95% CI)	<i>P</i> _{adj}
10	rs3729508	Intron 7*	A/G	GG/AG/AA	42/31/9	57.06/60.27/101.09	16.30 (4.28–28.31)	9.49E-03
12	rs6505469	Intron 5	T/A	AA/TA/TT	41/32/9	55.39/62.32/101.09	17.04 (5.03–29.06)	6.80E-03
15	rs3794767	Intron 2	T/C	CC/TC/TT	45/30/6	57.56/65.43/99.23	14.70 (1.50–27.9)	3.21E-02
20	rs8078340	Promoter	A/G	GG/AG/AA	41/30/12	65.82/63.27/55.81	-5.43 (-17.01–6.14)	0.3602
21	rs11080358	Promoter	A/G	GG/AG/AA	41/36/6	66.22/61.68/55.17	-5.99 (-19.34–7.35)	0.3813

^a Analysis was performed using the log-additive model, and the results were adjusted for age and gender (*P*_{adj}). Significant *P* values are highlighted in boldface. Introns denoted by asterisks are discussed in Table 2, footnote b.

^b Indicated as minor/major frequency alleles.

TABLE 6 Association of NOS2 SNPs with cerebral malaria^a

SNP no.	SNP reference	Gene region	Alleles ^b	Minor allele frequency	Genotypes	Genotype no.		CM vs UM	
						CM (n = 127)	UM (n = 142)	P _{adj}	OR (95% CI)
1	rs11653716	Intron 26	G/C	0.328	CC/GC-GG	66/60	59/82	0.07533	0.64 (0.39–1.05)
2	rs3794756	Intron 25	T/C	0.086	CC/TC-TT	106/20	121/21	0.92323	1.03 (0.53–2.03)
3	rs3729727	Intron 24	A/G	0.214	GG/AG-AA	77/50	87/55	0.86357	1.04 (0.63–1.72)
4	rs3729507	Intron 22	C/G	0.089	GG/CG-CC	105/22	121/21	0.70311	1.14 (0.59–2.2)
5	rs2872753	Intron 17	G/A	0.44	AA/GA-GG	45/81	38/104	0.12158	0.66 (0.39–1.12)
6	rs9282801	Intron 16*	T/G	0.271	GG/TG-TT	60/66	79/63	0.15144	1.43 (0.88–2.34)
7	rs3729720	Intron 14*	T/C	0.056	CC/TC-TT	109/18	131/11	0.06581	2.09 (0.94–4.66)
8	rs16966545	Intron 11	A/T	0.174	TT/AT-AA	81/45	99/42	0.25747	1.35 (0.8–2.28)
9	rs4795067	Intron 9	G/A	0.155	AA/GA-GG	87/39	107/34	0.26219	1.36 (0.79–2.35)
10	rs3729508	Intron 7*	A/G	0.188	GG/AG-AA	91/36	85/56	0.05503	0.61 (0.36–1.01)
11	rs3730017	Exon 7	T/C	0.24	CC/TC-TT	83/44	72/70	1.89E–02	0.56 (0.34–0.91)
12	rs6505469	Intron 5	T/A	0.167	AA/TA-TT	96/31	91/51	4.33E–02	0.58 (0.34–0.99)
13	rs16966563	Exon 4	C/T	0.236	TT/CT-CC	88/39	69/73	7.90E–04	0.43 (0.26–0.71)
14	rs3794766	Intron 2	T/C	0.153	CC/TC-TT	86/38	109/32	0.18941	1.45 (0.83–2.52)
15	rs3794767	Intron 2	T/C	0.167	CC/TC-TT	92/32	90/49	0.11435	0.65 (0.38–1.11)
16	rs3730013	Intron 1*	T/C	0.262	CC/TC-TT	66/61	79/63	0.65862	1.12 (0.69–1.81)
17	rs6505483	Intron 1	A/G	0.199	GG/AG-AA	79/48	90/50	0.55685	1.17 (0.7–1.95)
18	rs9282799	Promoter	T/C	0.034	CC/TC-TT	NA/NA	NA/NA	NA	NA
20	rs8078340	Promoter	A/G	0.269	GG/AG-AA	70/55	70/69	0.44726	0.83 (0.51–1.35)
21	rs11080358	Promoter	A/G	0.225	GG/AG-AA	79/47	82/59	0.52927	0.85 (0.52–1.4)

^a Association tests were performed using the dominant model, and the results were adjusted for age and gender (P_{adj}). Significant adjusted P values (P_{adj} < 0.05) are highlighted in boldface. rs16966563 (SNP 13) is still significant after Bonferroni correction (P = 1.58E–02). Introns denoted by asterisks are discussed in Table 2, footnote b. NA, not available.

^b Indicated as minor/major frequency alleles.

netic variation in the promoter region and demonstrated association signals influencing the outcome of infection (6, 8–12, 35). Furthermore, NOS2 promoter variants have been shown to drive NO production in malaria patients (11, 12, 36). In the present study, we identified two distinct regions in the NOS2 gene that govern NO plasma levels. The data indicate that the NOS2 promoter region significantly contributed to NO plasma levels in the absence of *Plasmodium* infection but did not significantly influence the clinical outcome of infection.

Remarkably, we found that in infected individuals the main region controlling NO plasma levels mapped within the cis-acting elements in this region that control NOS2 gene expression/activity are sensitized by *Plasmodium* infection through mechanisms yet to be elucidated. Our genetic study raises the possibility of a dual regimen of NOS2 gene expression control, entailing a promoter region that provides “basal” NO levels in the absence of infection and an intronic region that induces higher levels of NOS2 expression upon parasite infection.

The role of NO in malaria pathogenesis is controversial, and both detrimental and beneficial effects have been considered (37). It has been proposed that NO is produced at high levels to kill *Plasmodium* parasites and that elevated NO levels may impair neuronal signaling and generate oxidant damage and red blood cell damage that leads to anemia, contributing to the clinical features of malaria (38–40). Nevertheless, NO anti-inflammatory properties have been claimed to prevent cerebral malaria (3, 41), and epidemiological evidence is accumulating that NO plasma levels are decreased in cerebral malaria (6, 11, 12, 38).

Our genetic analysis in two different populations from the West coast of Africa entailed different designs to collect samples from healthy exposed individuals or clinical malaria cases but revealed conserved LD structure and comparable allelic frequencies

in the analyzed NOS2 region. The data cohesively suggest that genetic factors decreasing the NO level production provide decreased susceptibility to productive infection while genetic factors increasing NO plasma levels (namely, the T allele at SNP12, rs6505469) are associated with increased susceptibility to infection and protect against cerebral malaria. Since ascertainment of asymptomatic malaria cases may depend on the time point of collection, we also used pre-erythrocytic antibodies as an infection susceptibility marker. Finding that the presence of parasite DNA or antiparasite antibodies was associated with the same NOS2 SNP alleles reinforced the notion that susceptibility to infection is partially controlled by NOS2 variants.

Although we do not have access to measurements of local NO bioavailability, our data are consistent with the hypothesis that NO operates through anti-inflammatory properties to impair antiparasite responses, increasing susceptibility to infection, but also protecting against stronger inflammatory responses involved in progression to cerebral malaria. This complex pattern of NOS2 genetic control was revealed through combining results of the genetic analysis across the NOS2 gene in apparently healthy individuals and in clinical malaria cases. Although we analyzed individuals from two distinct African populations, further studies are needed to confirm these observations in other populations. Nevertheless, this work suggests that NO bioavailability attributable to NOS2 is subjected to a dual regimen of genetic control conditioned by infection. This may in part explain the apparently controversial results on the role of NOS2 polymorphisms and NO bioavailability in malaria.

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