

## Alleles 308A and 238A in the Tumor Necrosis Factor Alpha Gene Promoter Do Not Increase the Risk of Severe Malaria in Children with *Plasmodium falciparum* Infection in Mali<sup>∇</sup>

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**The hypothesis that tumor necrosis factor (TNF) aggravates malaria in children is supported by observations that TNF polymorphisms and high TNF levels have been associated with cerebral malaria. Nevertheless, severe malaria was not associated with polymorphisms located at positions –308A and –238A in the TNF alpha gene promoter or with a high TNF level in plasma in children from Bamako, Mali.**

The sequestration of parasitized and nonparasitized erythrocytes in the microvasculature and imbalances in the inflammatory response are thought to be crucial in the development of cerebral malaria (CM) (13, 16). This view is further supported by the report of associations between polymorphisms in ICAM-1 and CD-36 and severe malaria (11, 15, 20).

Among inflammatory cytokines, tumor necrosis factor (TNF) could affect the outcome of *Plasmodium falciparum* infection in several ways. TNF promotes fever, that may suppress parasite growth (18), and it also induces the expression of adhesion molecules and proinflammatory molecules. Lethal CM has been associated with a high level of TNF in serum (9, 12). The TNF alpha (TNFA)-308A/A genotype has been associated with CM in Gambian children (14, 15) but not in a Thai population (7), and it is unclear whether the polymorphism located at position –308A in TNF alpha (TNFA-308G/A) has a functional effect (1, 4). Observations in TNF<sup>-/-</sup> mice are not conclusive, and it seems that linkage disequilibrium between the TNF gene and another gene may account for the association between the TNF gene and severe *Plasmodium berghei* infection (5).

The present study tested the association of TNFA alleles with severe malaria in children hospitalized for either cerebral malaria (CM) or severe anemia (SA) at the Gabriel Touré Hospital in Bamako, Mali. The study included 1,105 participants clustered in 348 families and 42 unrelated children with uncomplicated malaria (UM) for the cytokine assay. The criteria used to define the CM and UM phenotypes were as previously described (3). The SA children were subjects with a thick blood film positive for *P. falciparum*, a packed cell volume of ≤15%, and no impaired consciousness.

The study was approved by the Ethics Committee of the Faculty of Medicine of Bamako. Informed consent was obtained from the parents, and 2 to 5 ml of peripheral blood was taken from each child and from both parents (or siblings if only one parent was available) of the children with severe malaria. Plasma and DNA were obtained as described previously (3).

**Lack of association between TNFA-308 alleles or TNFA-238 alleles and severe malaria.** We analyzed the TNFA-308G/A polymorphism in 208 families (136 CM and 72 SA) and the TNFA-238G/A polymorphism in 348 families (240 CM and 108 SA), with no parental inconsistencies (3). No significant deviation from the Hardy-Weinberg equilibrium was observed for both TNFA-308G/A and TNFA-238G/A in unrelated first-degree relatives of the CM groups ( $P = 0.308$  and  $P = 0.192$ ) and of the SA groups ( $P = 0.730$  and  $P = 0.170$ ) (Genepop program V3.4) (6). These alleles were not in linkage disequilibrium (LD) (Genepop program) (22).

TNFA-308G/A polymorphism in the promoter of the TNFA gene was determined as in the study by Wilson et al. (24) and Moukoko et al. (19). TNFA-308G was a common allele (frequency of 0.86). A total of 60 of the 136 children with CM and 31 of the 72 children with SA had at least one heterozygous parent (father and/or mother) and thus were informative for statistical analysis. FBAT V1.4 (8, 23) analysis showed that neither of the TNFA-308 alleles was preferentially associated with disease (Table 1).

The TNFA-238G/A polymorphism was determined as described previously (19). TNFA-238G was the common allele (0.95), and TNFA-238A was rare. A total of 32 of 240 children with CM and 11 of 108 children with SA had at least one heterozygous parent. Neither of the TNFA-238 alleles was preferentially transmitted from a heterozygous parent to an affected (CM or SA) child (Table 1).

The TNFA-376G/A polymorphism in the promoter of the TNFA gene was determined as described previously (19). TNFA-376A was in total LD with TNFA-238A, as described by Knight et al. (10). However, the TNFA-376A allele was rare,

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TABLE 1. Lack of association between TNFA-308G/A and TNFA-238G/A and severe malaria<sup>a</sup>

Phenotype	Allele	Allele frequency <sup>b</sup>	No. of informative families <sup>c</sup>	No. of transmitted alleles		Z <sup>f</sup>	P
				Observed <sup>d</sup>	Expected <sup>e</sup>		
CM	308G	0.86	60	89.0	86.0	0.74	0.46
	308A	0.14		31.0	34.0	-0.74	
SA	308G	0.86	31	44.0	43.5	0.174	0.86
	308A	0.14		18.0	18.5	-0.174	
CM	238G	0.96	32	48.0	46.3	0.56	0.57
	238A	0.04		16.0	17.7	-0.56	
SA	238G	0.97	11	19	16.5	1.51	0.13
	238A	0.03		3	5.5	-1.51	

<sup>a</sup> The analysis was carried out with FBAT using the additive model and a biallelic test.

<sup>b</sup> Calculated from the genotype of the parents.

<sup>c</sup> With at least one heterozygous parent.

<sup>d</sup> That is, the number of alleles transmitted from heterozygous parents to their affected child.

<sup>e</sup> That is, the number of alleles transmitted to an affected child on the hypothesis of no linkage disequilibrium.

<sup>f</sup>  $Z = [S - E(S)] / \sqrt{\text{Var}(S)}$ , where  $S$  is the test statistic (i.e., observed),  $E(S)$  is the expected value according to the null hypothesis (H0), and  $\text{Var}(S)$  is the variance of the statistic test according to H0.

and only four families were informative. Therefore, this allele could not be evaluated in the association test.

Since our results did not reach statistical significance, we performed power studies for the minor allele of the two SNPs, using PBAT v3.2, under the following assumptions: a type I error of 0.05, a population prevalence of the disease of 0.04 as derived previously (21), and an odds ratio of 3.6 as derived in an earlier study (10) for TNFA-308 and CM. PBAT yielded <0.50 power for the TNFA-238 polymorphism; TNFA-308A yielded 0.91 and 0.62 powers for CM and SA, respectively.

**TNF levels were low and similar between severe malaria and UM.** We measured the TNF levels by ELISA in the undiluted plasma (BD Pharmingen) of 42 children with UM, 58 children with CM, and 27 children with SA according to the manufacturer's instructions. Unmatched groups were compared by using the Mann-Whitney U test (SPSS 10.1 software), with  $P$  values of <0.05 being considered significant. The levels of TNF in the plasma of children with UM, CM, and SA were low (median 1pg/ml) and not significantly different (Fig. 1).

In conclusion, we found no evidence of an association between the TNFA-308 or TNFA-238 alleles and either CM or SA in Malian children in spite of a large number of informative families for TNFA-308. Our analysis also failed to detect significant differences in TNF plasma levels between children with CM or SA and control children. We should stress that definitive conclusions cannot be drawn with respect to the associations between TNFA-308 and SA, as well as TNFA-238 and SA or CM, since our data yielded a relatively low power to detect them. In contrast, there is much stronger evidence that, in Malian children, the association between TNFA-308 and CM is either absent or, at least, present at a much lower level than has been described in other African populations (10, 14, 15). This discrepancy may have several explanations. First, the causative allele might not be TNFA-308A (and probably not TNFA-238A) but some other alleles in LD. This LD would be lost in certain populations such as the Malian and Thai populations (7). Second, children recruited in The Gambia (14, 15) may have been affected by a more severe disease than those recruited in Mali. This is suggested by the higher case fatality rate of the Gambian children with CM compared to our study

participants. Thus, TNFA-308A may be specifically associated with a lethal outcome of CM. Histological observations on human brains have shown that TNF is produced in the small vessels of the brains of subjects who died from CM (2). Third, other polymorphisms located outside the TNFA gene may increase the risk of severe malaria in the Malian population, which is consistent with the low TNF levels in plasma in our patients. The lack of association between high TNF levels and

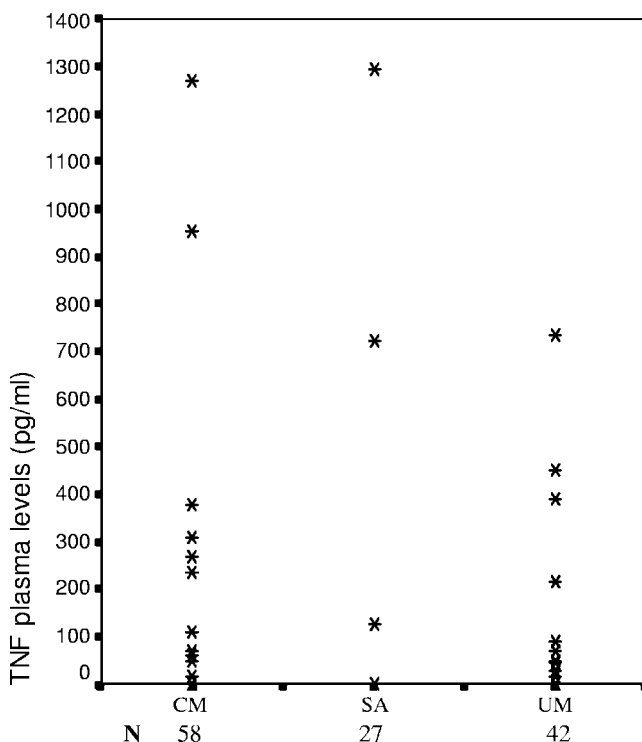


FIG. 1. TNF levels in plasma of the studied children. Each column represents the plasma level observed for each phenotype; each point represents data from one or several children. The detection threshold was 10 pg/ml. The data are presented as arithmetic means of duplicate values.

CM might also be explained by the fact that the level of TNF measured by ELISA may not reflect the amount of active TNF *in vivo*. Indeed, it has been reported that high levels of soluble TNF receptors are also found in the plasma of patients with malaria and may neutralize bioactive TNF (17). Finally, the studies that have demonstrated an association of TNFA alleles with severe malaria were case-control studies, whose results are notoriously affected by population stratification bias. Thus, spurious associations cannot be ruled out. This limitation is precluded here by using a family-based study design.

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