

Tumour necrosis factor alpha promoter polymorphism, TNF -238 is associated with severe clinical outcome of falciparum malaria in Ibadan southwest Nigeria

Subulade A Olaniyan^a, Olukemi K Amodu^a, Adekunle A Bakare^b, Marita Troye-Blomberg^c Olayemi O Omotade^a, Kirk A. Rockett^d, in collaboration with The MalariaGEN Consortium^d

^aInstitute of Child Health, College of Medicine, University of Ibadan, Ibadan, Oyo, Nigeria

^bCell Biology & Genetics Unit, Department of Zoology, University of Ibadan, Ibadan, Oyo, Nigeria

^cDepartment of Immunology, Wenner-Gren Institute, Stockholm University, Stockholm, Sweden

^dWellcome Trust Centre for Human Genetics, University of Oxford, Oxford OX3 7BN, UK

Author for Correspondence: Dr. Olukemi K. Amodu
Institute of Child Health
College of Medicine,
University of Ibadan
Email: amkemi@hotmail.com
Tel: 234-0-8076454679
Fax: 234-2-2411768

Abstract

Tumour necrosis factor (TNF) - α has been shown to play an important role in the pathogenesis of falciparum malaria. Two TNF promoter polymorphisms, TNF-308 and TNF-238 have been associated with differential activity and production of TNF. In order to investigate the association between TNF-308 and TNF-238 and the clinical outcome of malaria in a Nigerian population, the two TNF polymorphisms were analyzed using Sequenom iPLEX Platform. A total of 782 children; 283 children with uncomplicated malaria, 255 children with severe malaria and 244 children with asymptomatic infection (controls) were studied. The distribution of TNF-308 and TNF-238 genotypes were consistent with the Hardy-Weinberg equilibrium. Distribution of both TNF polymorphisms differed significantly across all clinical groups (TNF-308: $p=0.007$; TNF-238: $p = 0.001$). Further tests for association with severe malaria using genotype models controlling for age, parasitaemia and HbAS showed a significant association of the TNF-238 polymorphism with susceptibility to severe malaria (95% CI=1.43 – 6.02, OR=2.94, $p=0.003237$) The GG genotype of TNF-238 significantly increased the risk of developing cerebral malaria from asymptomatic malaria and uncomplicated malaria (95% CI = 1.99 – 18.17, OR = 6.02, $p < 0.001$ and 95% CI = 1.78 – 8.23, OR = 3.84, $p < 0.001$ respectively). No significant association was found between TNF-308 and malaria outcome. These results show the genetic association of TNF-238 in the clinical outcome of malaria in Ibadan, southwest Nigeria. These findings add support to the role of TNF in the outcome of malaria infection. Further large scale studies across multiple malaria endemic populations will be required to determine the specific roles of TNF-308 and TNF-238 in the outcome of falciparum malaria infection.

47

48 **Keywords:** Cytokine, TNF, severe malaria, promoter polymorphism

49

1.0 Introduction

Malaria is a complex disease and the host genetic factors play a critical role in the control of the internal environment which the parasite faces during malaria infection (Mergani et al., 2010). Cytokines are a major component of the host genetic factors; they play a key role in the regulation of the host immune response (both innate and adaptive responses) in malaria infection (Clark et al., 2009; Israelsson et al., 2011). Tumour necrosis factor alpha (TNF), a very important multifunctional pro-inflammatory cytokine, is produced by a wide range of cells including macrophages, neutrophils, activated lymphocytes and natural killer cells (Mergani et al., 2010). In malaria, TNF plays a dual role in the pathogenesis and control of parasitemia (Flori et al., 2005). Several studies have suggested that TNF is the main mediator of secondary complications during severe falciparum malaria (Rockett et al., 2014; Clark et al., 2009; Kremsner et al., 1995).

A number of single nucleotide polymorphisms (SNPs) are found in the promoter region of TNF located at nucleotide positions (relative to the transcription initiation site) -238 (TNF 238), -244 (TNF 244), -308 (TNF 308), -857 (TNF 857), -863 (TNF 863) -1031 (TNF 1031) and -376 (TNF 376), (Clark et al., 2009; Mergani et al., 2010). Most of these SNPs have been associated with inter-individual differences in TNF serum and plasma levels as well as differences in the outcome of malaria infection (Ahmad et al., 2003; McGuire et al., 1994; Santovito et al., 2012). Studies have shown that two of these TNF polymorphisms, TNF -308 and TNF -238 are highly relevant in the production of TNF (D'Alfonso & Richiardi, 1994; Ozhan et al., 2010) and have been associated with

differential activity and production of TNF (Ahmad et al., 2003; Higuchi et al., 1998). TNF -308 has been linked with increased transcriptional activity and production of TNF (Ahmad et al., 2003; Wilson et al., 1997). The alleles (A and G) of the TNF -308 promoter gene have been shown to influence the production of TNF differently. The TNF -308A has been found to activate the production of TNF more strongly than TNF -308G in a human B-cell line (Abraham & Kroeger, 1999). Furthermore, studies have shown the correlation of TNF polymorphisms with the induction of antibody levels to *P. falciparum* antigens such as AMA1, CSP, MSP1, MSP2 and total IgE (Maiga et al., 2014). TNF -308A homozygous individuals (AA) have been shown to have higher circulating TNF levels than TNF -308G homozygous (GG) individuals (Bouma et al., 1996). However, the reports of the influence of TNF -238 on the transcriptional regulation of TNF have been conflicting (Drouet et al., 1991; Kaluza et al., 2000; Pociot et al., 1995; Zhang et al., 2011).

These TNF polymorphisms, TNF-308 (rs1800629) and TNF-238 (rs361525) have been associated with different clinical outcomes of malaria in different African populations (Carpenter et al., 2009; Clark et al., 2009; McGuire et al., 1999, 1994; Mombo et al., 2003). The association between a particular SNP and the outcome of an infection has been shown to be dependent on the ethnic background of the population being studied (Laishram et al., 2012), especially in African populations that are highly characterized by genetic diversity. Hence, the association between a particular SNP and the outcome of an infection may vary across populations. Nigeria still has one of the highest malaria-associated death toll in the world with 39% of global malaria deaths accounted for by

Nigeria & the Democratic Republic of Congo (WHO, 2014). A few studies have been carried out to investigate the role of TNF in the pathogenesis of malaria in different parts of Nigeria (Nmorsi et al., 2010; Noone et al., 2013). However, these studies focused mainly on plasma and serum TNF levels/concentration in association with the outcome of malaria infection. Our study will be the first to analyze the genetic association between the different genotypes of TNF -308 and TNF -238 and falciparum malaria in Nigeria. We therefore investigated the association between TNF -308G/A and TNF -238G/A alleles and the clinical outcome of malaria, testing the hypothesis that TNF-308G/A and TNF -238G/A are associated with the outcome of severe malaria in Ibadan, Nigeria.

2.0 Materials and Methods

2.1. Study area

A total of 782 children [255 with severe malaria (105 and 150 children with severe malaria anaemia and cerebral malaria respectively), 283 with uncomplicated malaria, and 244 with asymptomatic malaria] were recruited from Children's Emergency ward and Children Out-patient Clinic of three hospitals in Ibadan southwest Nigeria, a region of hyperendemic malaria transmission. The 244 asymptomatic controls were recruited from secondary schools in the same locality, the catchment area of the hospitals from which the uncomplicated and severe malaria cases were recruited. Almost all the children recruited into this study (93%) were from the Yoruba ethnic group, the major ethnic populace of the study area. The children were categorized into asymptomatic malaria, uncomplicated malaria and severe malaria groups based on World Health

Organization criteria (WHO, 2010). Asymptomatic malaria was defined as presence of asexual *P. falciparum* in peripheral thick blood smears, an axillary temperature of <37.5°C and an absence of malaria-related symptoms. Uncomplicated malaria was defined as presence of asexual parasitaemia and a temperature of >37.5°C without severe malaria symptoms. Severe malaria was defined as presence of asexual parasitaemia, packed cell volume of <15% and unrousable coma which persisted for more than 30 minutes after a seizure. Severe malaria was further sub-categorized into severe malaria anaemia (defined as packed cell volume of <15%) and cerebral malaria (unrousable coma which persisted for more than 30 minutes after a convulsion, Blantyre score of ≤2). The study was approved by the Joint University of Ibadan and University College Hospital Ethical Committee (UI/IRC/06/0034). Informed consent was obtained from the parents of all children recruited for the study. The samples were collected within three malaria transmission seasons 2007 – 2010 and the sample processing (DNA extraction and genotyping) and analysis of data was done 2011 - 2012.

2.2. Collection of blood samples and DNA extraction

Five milliliters of venous blood was collected from each child into sterile EDTA tubes. Malaria parasites were examined on 5% Giemsa-stained thick and thin blood smears. The parasites were counted against 200 white blood cells and the parasite densities were calculated based on an assumed total white blood cell of 8,000/μL (WHO, 2010). All giemsa-stained thick and thin blood smears were independently viewed by two experienced microscopists in the Institute of Child Health, College of Medicine, University of Ibadan. The results were compared and any sample with discordant

results was viewed by a third experienced microscopist. In addition, some of the samples were picked randomly to validate the microscopy results using species-specific PCR.

DNA was extracted from whole blood using the Nucleon BACC 2 Kit (Gen-Probe Life Sciences, Manchester) following manufacturer's instructions with slight modifications. The DNA samples were shipped frozen to Oxford University. Some of the samples were again picked randomly to validate the microscopy results using species-specific PCR to exclude co-infections. Genotyping was undertaken by the MalariaGEN Consortium Resource Centre using the Sequenom® iPLEX Gold platform (Dunstan et al., 2012; Toure et al., 2012).

2.3. Statistical analysis

Statistical analysis was performed using SPSS ® 16.0 software. Haploview 4.2 was used to calculate allele frequencies and to test for Hardy-Weinberg equilibrium. The Hardy-Weinberg analysis was performed in the control group. Chi squared statistics was used to examine differences between groups. Each TNF SNP was tested for association by comparing between different groups: i) asymptomatic malaria group with severe malaria group; ii) asymptomatic group with cerebral malaria group; iii) asymptomatic malaria group with severe malaria anaemia group (not shown); iv) uncomplicated malaria group with severe malaria group; v) uncomplicated malaria group with cerebral malaria group; vi) uncomplicated malaria group with severe malarial anaemia group. The association analysis was done using logistic regression models with adjustment for age, parasite density and HbS. Statistical significance was defined

as $p < 0.005$ because our analysis involved 10 comparisons (2 SNPs examined under 5 genetic models), this Bonferroni correction threshold conservatively corrects for the multiple comparisons. Each SNP was modeled assuming several related genetic models with respect to the derived allele: i) recessive (AA vs AG/GG); ii) dominant (GG vs AG/AA); iii) heterozygote (AG vs AA/GG); iv) additive (0[GG]: 1[AG] :2[AA]); and genotypic models. The additive model imposes a structure in which each additional copy of the variant allele increases the response by the same amount. In other words, the additive effect of AG is half of the difference between the mean of all cases that are homozygous for one version of the allele.

3.0 Results

The study population comprised of 432 males (55.2%). The mean age (in months) of the children in the asymptomatic, uncomplicated and severe malaria groups were 52.9 ± 32.7 , 46.6 ± 30.7 , and 39.6 ± 25.2 respectively. Table 1 shows the demographic and clinical characteristics for the asymptomatic, uncomplicated and severe malaria groups in Ibadan southwest Nigeria.

Table 1: Demographic and clinical parameters of children from Ibadan southwestern Nigeria

Parameters	Asymptomatic malaria	Uncomplicated malaria	Severe malaria	p value
Number of participants	244	283	255	
Gender				
Male	133	152	147	
Female	111	131	108	
Mean age (months)	52.9 ± 32.7	46.6 ± 30.7	39.6 ± 25.2	0.001
Mean packed cell volume (%)	33.4 ± 6.11	31.5 ± 4.5	21.3 ± 8.5	<0.001*
Mean parasite density (/µl)	440 ± 0.7	941 ± 0.9	1940 ± 1.1	<0.001*
Mean temperature	36.5 ± 0.9	37.6 ± 0.7	38.1 ± 0.8	<0.001*
Hb AS	0.16	0.15	0.07	0.067
Hb S allele	0.10	0.09	0.06	0.08

*p values less than 0.001

The mean body temperature was highest in the severe malaria group with a temperature of 38.1 ± 0.8°C while it was lowest in the asymptomatic control group with a temperature of 36.5 ± 0.9°C. The proportion of females was similar in each group (45.5%, 46.3% and 42.4% of asymptomatic, uncomplicated and severe malaria cases respectively). Both cytokine SNPs were found in Hardy-Weinberg equilibrium. The genotype and allele frequencies of TNF- α -308G/A and TNF- α -238G/A in the study population are presented in Table 2.

Table 2: Genotype and allele frequencies of TNF -308 and TNF -238 polymorphisms in asymptomatic children from Ibadan southwest Nigeria

TNF genotype	Genotype frequency	Allele	Allele frequency	^a p HWE	^b MAF
TNF -308					
GG	0.82	A	0.096	0.6	0.096
AG	0.17	G	0.903		
AA	0.01				
TNF -238					
GG	0.98	A	0.01	0.3	0.01
AG	0.02	G	0.99		
AA	0.0				

^aThe p_{HWE} is the Hardy-Weinberg equilibrium p value, which is the probability that its deviation from Hardy-Weinberg equilibrium could be explained by chance. The two SNPs were tested for Hardy-Weinberg equilibrium (HWE) using Haploview 4.2 software and a cut-off p value of 0.001 was set.

^bMAF, minor allelic frequency

The allele frequencies were 0.903 for TNF -308G and 0.096 for TNF -308A. The allele frequency of TNF -238G was 0.99, while it was 0.01 for TNF -238A. Table 3 shows the

212 distribution of the TNF -308G/A and TNF -238G/A genotypes between the
 213 asymptomatic, uncomplicated and severe malaria groups. Comparing the three groups
 214 together using chi-squared test showed a significant difference in the genotypic
 215 distribution of the two TNF SNPs (Table 3) between the malaria groups.

216 Table 3: Differences in TNF -308 and TNF -238 genotype frequencies across malaria
 217 groups in children from Ibadan southwest Nigeria

TNF	Asymptomatic	Uncomplicated	Severe	P _A	P _B	P _C	P _D
genotype	malaria control	malaria cases	malaria cases				
	(N=244), n(%)	(N=283), n(%)	(N = 255), n(%)				
<hr/>							
TNF-							
308A/G							
AA	3 (1.2)	5 (1.8)	-	0.007	0.008	0.002	0.567
AG	41 (16.8)	39 (13.8)	20 (7.8)				
GG	200 (82.0)	239 (84.5)	235 (92.2)				
Allele							
frequency							
A	0.096	0.087	0.039				
G	0.903	0.913	0.961				
 TNF-							
238A/G							
AA	-	1 (0.4)	4 (1.6)	0.001	0.018	0.001	0.343
AG	6 (2.5)	12 (4.2)	24 (9.4)				
GG	238 (97.5)	270 (95.4)	227 (89.0)				

Allele			
frequency			
A	0.01	0.03	0.06
G	0.99	0.97	0.94

n, number of individuals positive for the genotype of interest; p, chi squared p-values comparing (A) all 3 groups, (B) uncomplicated cases vs severe malaria cases, (C) asymptomatic controls vs severe malaria cases and (D) asymptomatic controls vs uncomplicated cases

Pairwise comparison of groups (of uncomplicated cases vs severe malaria cases and asymptomatic controls vs severe malaria cases) also showed significant differences in the distribution of the TNF genotypes at the two loci (Table 3). Exploratory analyses comparing the genotype frequencies of the TNF SNPs among asymptomatic controls and severe malaria sub-groups showed a significant difference in the distribution of both TNF genotypes: severe malarial anaemia- ASM vs SMA [TNF 308 - $p=0.019$, TNF 238 - $p=0.049$]; and cerebral malaria - ASM vs CM [TNF 308 - $p=0.026$, TNF 238 - $p<0.001$ (data not shown). However, when compared between each severe malaria sub-group (severe malaria anaemia and cerebral malaria) and uncomplicated malaria cases, the genotype frequencies did not significantly differ (data not shown).

Further analysis using genotype-based tests showed associations between the two TNF SNPs and malaria outcome in all the comparison groups (Table 4). The genotypic proportions of TNF -308 did not vary significantly between different groups under the different genetic models. In contrast, significant associations were observed for TNF -238 in the asymptomatic vs cerebral malaria groups (dominant: OR = 6.02, 95% CI =

1.99 – 18.17, $p = 0.00145$; additive: OR = 5.53, 95% CI = 1.92 – 15.8, $p = 0.001506$);
uncomplicated vs severe malaria groups (additive: OR = 2.94, 95% CI = 1.43 – 6.02, p
= 0.003237); uncomplicated vs cerebral malaria groups (dominant: OR = 3.48, 95% CI =
1.78 – 7.63, $p = 0.004324$). No significant differences were found when TNF -238 was
analysed in the asymptomatic vs severe malaria groups.

We performed power analysis to estimate how much power our study has to identify
true association with minor allele frequency of 3.0%, population risk of 0.5%, OR ≥ 3.5
and sample size of at least 200 in each of the comparison groups. Our calculations
indicate we have 87.7% and 83.7% power to detect true associations in 2-sided test
with Type I error rate of 0.005.

252 Table 4: Genetic analysis models of the TNF -308 and TNF -238 SNPs in children from Ibadan southwest Nigeria

Comparison		SNP		Ancestral	Derived	Minor	Genotype	OR (95% CI)	p value
Groups				allele	allele	allele	model		
^a ASM	^b SM	rs1800629	-TNF-308	G	A	A	Recessive	1.21E-09 (0 - ∞) ^e	0.999
							Dominant	0.46 (0.23 - 0.95)	0.034
							Heterozygote	0.47 (0.23 – 0.96)	0.040
							Additive	0.46 (0.23 – 0.96)	0.032
							Genotypic	0.49 (0.24 – 1.02)	0.060
ASM	^c CM	rs1800629	-TNF-308	G	A	A	Recessive	1.64E-09 (0 - ∞) ^e	0.999
							Dominant	0.45 (0.21 – 0.96)	0.040
							Heterozygote	0.47 (0.21 – 1.06)	0.070
							Additive	0.45 (0.21 – 0.95)	0.036
							Genotypic	0.47 (0.21 – 1.07)	0.070
^d UM	SM	rs1800629	-TNF-308	G	A	A	Recessive	4.58E-10 (0 - ∞) ^e	0.998
							Dominant	0.49 (0.27 – 0.91)	0.022
							Heterozygote	0.56 (0.30 – 1.03)	0.060

UM	CM	rs1800629 -TNF-308	G	A	A	Additive	0.48 (0.27 – 0.84)	0.011
						Genotypic	0.55 (0.32 – 1.05)	0.700
						Recessive	5.75E-10 (0 - ∞) ^e	0.998
						Dominant	0.46 (0.20 – 0.89)	0.022
						Heterozygote	0.58 (0.29 – 1.18)	0.140
ASM	SM	rs361525 -TNF-238	G	A	A	Additive	0.45 (0.24 -0.85)	0.012
						Genotypic	0.61 (0.30 – 1.23)	0.160
						Recessive	1.25E+09 (0 - ∞) ^e	0.998
						Dominant	4.64 (1.56 - 13.8)	0.006
						Heterozygote	3.87 (1.26 – 11.8)	0.020
ASM	CM	rs361525 -TNF-238	G	A	A	Additive	4.54 (1.55 – 12.3)	0.005
						Genotypic	3.68 (1.21 – 11.2)	0.020
						Recessive	1.66E+09 (0 - ∞) ^e	0.998
						Dominant	6.02 (1.99 – 18.2)	0.001
						Heterozygote	4.56 (1.47 – 14.3)	0.009
						Additive	5.53 (1.92 – 15.8)	0.001

							Genotypic	4.60 (1.48 – 14.3)	0.008
UM	SM	rs361525	-TNF-238	G	A	A	Recessive	1.18E+09 (0 - ∞) ^e	0.998
							Dominant	2.98 (1.38 – 6.42)	0.005
							Heterozygote	2.50 (1.13 – 5.50)	0.020
							Additive	2.94 (1.43 – 6.02)	0.003
							Genotypic	2.50 (1.10 – 5.44)	0.030
UM	CM	rs361525	-TNF-238	G	A	A	Recessive	2,36E+09 (0 - ∞) ^e	0.998
							Dominant	3.84 (1.78 – 8.23)	0.0006
							Heterozygote	3.23 (1.42 – 7.36)	0.005
							Additive	3.69 (1.78 – 7.24)	0.0004
							Genotypic	3.15 (1.38 – 7.24)	0.007

253 ^aAsymptomatic malaria, ^bsevere malaria, ^ccerebral malaria, ^duncomplicated malaria, ^e95% CI are shown as infinity for divergent

254 estimates due to limited sample size

255 Significant associations (P < 0.005) are highlighted in bold

256

257

258

259

260

4.0 Discussion

The onset of severe malaria in children in this study coincides with previous reports of increased susceptibility to severe malaria in the first three to five years of life (Mergani et al., 2010; Perlmann & Troye-Blomberg, 2002). In malaria-endemic regions like Ibadan in Nigeria, younger children are particularly susceptible to severe malaria. As they grow older with increased exposure to episodes of severe malaria, these children develop active immunity to the severe form of the infection.

Cytokine polymorphisms have been known to be associated with the outcome of malaria infection. Tumour necrosis factor (TNF) polymorphisms have been implicated in the susceptibility/protection to malaria in several African populations (Jallow et al., 2009). This study was carried out to investigate the role of two TNF SNPs, TNF -308 and -238 in the clinical outcome of malaria. The distribution of the two TNF polymorphisms, -308 and -238 were consistent with Hardy-Weinberg equilibrium. Both TNF polymorphisms were found at significantly different frequencies across the malaria groups, signifying an association with malaria outcome. Further analysis using genotypic (i.e., each of the three possible is treated as different from any other) tests, adjusting for age, parasite density and HbS showed a strong association between TNF -238 and malaria outcome. However, we did not find any association between TNF -308 and malaria outcome. Previous studies in central Sudan, Gabon and Thailand reported similar findings between TNF -308 and malaria outcome (Hananantachai et al., 2001; Mergani et al., 2010; Meyer et al., 2002). Other studies however, found an association between the homozygous AA genotype of the TNF -308 polymorphism and severe

malaria (Brinkman et al., 1995; Knight & Kwiakowski, 1999). In The Gambia, children who were homozygous for the TNF -308A allele had a sevenfold increased risk for severe malaria; a similar association was found in Sri Lanka, where the homozygous TNF -308A genotype was associated with severe malarial infections, compared to uncomplicated malaria infections (McGuire et al., 1999, 1994; Wattavidanage et al., 1999).

We found TNF -238G/A to be associated with progression of infection from uncomplicated malaria to severe malaria. This association of TNF -238 and the progression of malaria infection from uncomplicated to severe malaria remained significant only in the cerebral malaria group. Specifically, the GG genotype was associated with an increased risk of developing cerebral malaria from uncomplicated malaria. Furthermore, the frequencies of GG individuals were higher in the cerebral malaria group than in the uncomplicated malaria group. This suggests that the association found between the TNF -238 locus and the progression of malaria infection and severe malaria is specific only to cerebral malaria and not to the severe malaria anaemia. Previous studies have showed differential associations between TNF -238 and the clinical outcome of malaria. In a Kenyan population, TNF -238A allele was associated with relative protection from cerebral malaria while a Gambian study found an association between the -238A allele and susceptibility to severe malaria but not to cerebral malaria (Knight et al., 1999; McGuire et al., 1999; Mombo et al., 2003). These findings suggest that TNF -238 may play an important role in regulating TNF production. Functional studies on the effect/role of TNF -238 on transcriptional regulation of TNF

have been conflicting. Pociot et al (1995) and Drouet et al. (1991) reported a lack of association between TNF -238 and transcription levels of TNF while Kaluza et al. (2010) found out that TNF -238 reduced transcriptional activity (Drouet et al., 1991; Kaluza et al., 2000; Pociot et al., 1995). Although we found associations between TNF -238 genotypes and the progression of clinical malaria, we think that the clinical outcome of malaria may be linked with other genes or gene polymorphisms lying within the MHC region where the TNF gene is located.

TNF -308A homozygous individuals (AA) have been shown to have higher circulating TNF levels than TNF -308G homozygous (GG) individuals (Bouma et al., 1996). Sohali et al (2008) also found that the TNF -308 G/A positions and at the 1031 T/C positions correlated significantly with the TNF-alpha levels in vivax malaria. The absence of immunoassay data that could have been generated by measuring the circulating TNF levels in the children in the different clinical malaria groups was a limitation in demonstrating the role of TNF in the clinical outcome of malaria in this study.

Taken together, we found no association between the TNF -308 polymorphism and the clinical outcome of malaria, while we found the TNF -238 polymorphism, specifically the GG genotype to be associated with the progression of malaria from asymptomatic and uncomplicated forms to cerebral malaria, in contrast to the associations reported from other populations. These disparities in association could be as a result of our study design. It is important to note that the present study is not a simple case-control study (with healthy controls and malaria cases), but one looking at the progression of malaria

infection by comparing the TNF polymorphisms across clinical groups. The control group in this study was the asymptomatic malaria group while the cases were the uncomplicated and severe malaria groups. Hence, the slight difference in design could account for the differences in association of the TNF polymorphisms with malaria outcome in our study and previous association studies in other populations. These disparities of association could also be due to genetic heterogeneity in our study population. African populations have been known to be characterized by a high degree of genetic diversity. Genetic diversity is usually generated as a result of uniqueness in population structure and ethnic diversity which could lead to a local effect of the TNF polymorphisms on the outcome of malaria in our study population.

5.0 Conclusions

Overall, this study tested the association between two tumour necrosis factor alpha polymorphisms, TNF -308 and TNF -238 and falciparum malaria in a Nigerian population and found TNF -238 to be associated with malaria outcome. These findings add to support the current view/belief that TNF plays an important role in malaria pathogenesis and that it influences the clinical outcome of malaria. Our findings further support the heterogeneity of TNF alleles and their differential associations with malaria outcome in African populations.

Conflicting interests

The authors declare that they have no conflicting interests.

353

354 **Acknowledgements**

355 The authors are grateful to the staff, nurses and doctors of the hospitals for their care of
356 the patients. We are also grateful to the field and laboratory staff of the project;
357 Olajumoke Imolehin, Funmilola Ajayi, Folakemi Amodu and Mojisola Oyeniyi. We thank
358 Anna Jeffreys, Kate Rowlands and Christina Hubbart in the MalariaGEN resource
359 centre for their assistance in DNA handling and genotyping. The research leading to
360 these results has received funding from the European Community under grant
361 agreement LSHP-CT-2004-503578 and Seventh Framework Programme (FP7/2007-
362 2013) under grant agreement N° 242095. MalariaGEN is supported by the Wellcome
363 Trust (WT077383/Z/05/Z) and by the Foundation for the National Institutes of Health
364 (566) as part of the Bill & Melinda Gates Grand Challenges in Global Health Initiative.
365 The Resource Centre for Genomic Epidemiology of Malaria is supported by the
366 Wellcome Trust (090770/Z/09/Z). Support was also provided by the Medical Research
367 Council (G0600718). The Wellcome Trust also provides core awards to the Wellcome
368 Trust Centre for Human Genetics (090532/Z/09/Z) and to the Wellcome Trust Sanger
369 Institute (098051).

370

371 **References**

372 Abraham, L. J., & Kroeger, K. M. (1999). Impact of the -308 TNF promoter
373 polymorphism on the transcriptional regulation of the TNF gene: relevance to
374 disease. *Journal of Leukocyte Biology*, 66, 562–566.

375 Ahmad, T., Wallace, G. R., James, T., Neville, M., Bunce, M., Mulcahy-Hawes, K.
 376 Jewell, D. P. (2003). Mapping the HLA association in Behçet's disease: a role for
 377 tumor necrosis factor polymorphisms? *Arthritis and Rheumatism*, 48, 807–813.
 378 doi:10.1002/art.10815

379 Bouma, G., Crusius, J. B. A., Pool, M. O., Kolkman, J. J., Von Blomberg, B. M. E.,
 380 Kostense, P. J., ... S. G. M., Penã, A. S. (1996). Secretion of tumor necrosis factor
 381 α and lymphotoxin α in relation to polymorphisms in the TNF genes and HLA-DR
 382 alleles. Relevance for inflammatory bowel disease. *Scand. J. Immunol.*, 43, 456–
 383 463.

384 Brinkman, B. M., Zuijdeest, D., Kaijzel, E. L., Breedveld, F. C., & Verweij, C. L. (1995).
 385 Relevance of the tumor necrosis factor α (TNF α) - 308 promoter
 386 polymorphism in TNF α gene regulation. *Journal of Inflammation*, 46(1), 32–41.

387 Carpenter, D., Rooth, I., Färnert, A., Abushama, H., Quinnell, R. J., & Shaw, M.-A.
 388 (2009). Genetics of susceptibility to malaria related phenotypes. *Infection, Genetics*
 389 *and Evolution : Journal of Molecular Epidemiology and Evolutionary Genetics in*
 390 *Infectious Diseases*, 9(1), 97–103. doi:10.1016/j.meegid.2008.10.008

391 Clark, T. G., Diakite, M., Auburn, S., Campino, S., Andrew, E., Green, A., Kwiatkowski,
 392 D. P. (2009). Tumor Necrosis Factor and Lymphotoxin- α Polymorphisms and
 393 Severe Malaria in African Populations. *Journal of Infectious Diseases*, 199(4), 569–
 394 575. doi:10.1086/596320.Tumor

395 D'Alfonso, S., & Richiardi, P. . (1994). A polymorphic variation in a putative regulation
396 box of the TNFA promoter region. *Immunogenetics*, 39(2), 150–154.

397 Drouet, C., Shakhov, A. N., & Jongeneel, C. V. (1991). Enhancers and transcription
398 factors controlling the inducibility of the tumor necrosis factor-alpha promoter in
399 primary macrophages. *J Immunol*, 147(1694–1700).

400 Dunstan, S. J., Rockett, K. a, Quyen, N. T. N., Teo, Y. Y., Thai, C. Q., Hang, N. T.,
401 Hien, T. T. (2012). Variation in human genes encoding adhesion and
402 proinflammatory molecules are associated with severe malaria in the Vietnamese.
403 *Genes and Immunity*, 13(6), 503–8. doi:10.1038/gene.2012.25

404 Flori, L., Delahaye, N. F., Iraqi, F. a, Hernandez-Valladares, M., Fumoux, F., & Rihet, P.
405 (2005). TNF as a malaria candidate gene: polymorphism-screening and family-
406 based association analysis of mild malaria attack and parasitemia in Burkina Faso.
407 *Genes and Immunity*, 6(6), 472–80. doi:10.1038/sj.gene.6364231

408 Hananantachai, H., Patarapotikul, J., Looareesuwan, S., Ohashi, J., Naka, I., &
409 Tokunaga, K. (2001). Lack of association of -308A/G TNFA promoter and 196R/M
410 TNFR2 polymorphisms with disease severity in Thai adult malaria patients.
411 *American Journal of Medical Genetics*, 102(4), 391–392.

412 Higuchi, T., Seki, N., Kamizono, S., Yamada, A., Kimura, A., Kato, H., & Itoh, K. (1998).
413 Polymorphism of the 5'-flanking region of the human tumor necrosis factor (TNF)-
414 alpha gene in Japanese. *Tissue Antigens*, 51, 605–612.

415 Israelsson, E., Maiga, B., Kearsley, S., Dolo, A., Homann, M. V., Doumbo, O. K., ...
 416 Berzins, K. (2011). Cytokine gene haplotypes with a potential effect on
 417 susceptibility to malaria in sympatric ethnic groups in Mali. *Infection, Genetics and*
 418 *Evolution : Journal of Molecular Epidemiology and Evolutionary Genetics in*
 419 *Infectious Diseases*, 11(7), 1608–15. doi:10.1016/j.meegid.2011.05.021

420 Jallow, M., Teo, Y. Y., Small, K. S., Rockett, K. a, Deloukas, P., Clark, T. G.,
 421 Kwiatkowski, D. P. (2009). Genome-wide and fine-resolution association analysis of
 422 malaria in West Africa. *Nature Genetics*, 41(6), 657–65. doi:10.1038/ng.388

423 Kaluza, W., Reuss, E., Grossmann, S., Hug, R., Schopf, R. E., & Galle, P. R. (2000).
 424 Different transcriptional activity and in vitro TNF-alpha production in psoriasis
 425 patients carrying the TNF-alpha 238A promoter polymorphism. *J Invest Dermatol*,
 426 114, 1180–1183.

427 Knight, J. C., & Kwiatkowski D. (1999). Inherited variability of tumor necrosis factor and
 428 production susceptibility to infectious disease. *Proceedings of the Association of*
 429 *American Physicians*, 111(4), 290–298.

430 Knight, J., Udalova, I., Hill, A., Greenwood, B., Peshu, N., Marsh, K., & Kwiatkowski, D.
 431 (1999). A polymorphism that affects OCT-1 binding to the TNF promoter region is
 432 associated with severe malaria. *Nat Genet*, 22(2), 145–150.

433 Kremsner, P. G., Winkler, S., Brandts, C., Wildling, E., Jenne, L., Graninger, W., Grau,
 434 G. E. (1995). Prediction of accelerated cure in Plasmodium falciparum malaria by

435 the elevated capacity of tumor necrosis factor production. *The American Journal of*
436 *Tropical Medicine and Hygiene*, 53, 532–538.

437 Laishram, D. D., Sutton, P. L., Nanda, N., Sharma, V. L., Sobti, R. C., Carlton, J. M., &
438 Joshi, H. (2012). The complexities of malaria disease manifestations with a focus
439 on asymptomatic malaria. *Malaria Journal*. doi:10.1186/1475-2875-11-29.

440 Maiga B., Dolo A., Touré O., Dara V., Tapily A., Campino S., Sepulveda N., Risley P.,
441 Silva N., Corran P., Rockett K. A., Kwiatkowski D., MalariaGEN Consortium., Clark
442 TG., TroyeBlomberg M., Doumbo O K. (2014) Human candidate polymorphisms in
443 sympatric ethnic groups differing in malaria susceptibility in Mali. *PLoSOne*.
444 8(10):e75675. doi: 10.1371/journal.pone.0075675.

445 McGuire, W., Hill, A. V, Allsopp, C. E., Greenwood, B. M., & Kwiatkowski, D. (1994).
446 Variation in the TNF-alpha promoter region associated with susceptibility to
447 cerebral malaria. *Nature*, 371, 508–510. doi:10.1038/371508a0

448 McGuire, W., Knight, J. C., Hill, A. V., Allsopp, C. E., Greenwood, B. M., & Kwiatkowski,
449 D. (1999). Severe malarial anemia and cerebral malaria are associated with
450 different tumor necrosis factor promoter alleles. *The Journal of Infectious Diseases*,
451 179, 287–290. doi:10.1086/314533

452 Mergani, A., Khamis, A. H., Haboor, A. B., Hashim, E., Gumma, M., Awadelseed, B.,
453 Elwali, N. M. A. (2010). Lack of association between - 308 tumor necrosis factor
454 polymorphism and susceptibility to cerebral malaria among Central Sudanese

455 children. *International Journal of Genetics and Molecular Biology*, 2.5(May 2010),
 456 67–71.

457 Meyer, C. G., May, J., Luty, A. J., Lell, B., & Kremsner, P. G. (2002). TNFalpha-308A
 458 associated with shorter intervals of Plasmodium falciparum reinfections. *Tissue*
 459 *Antigens*, 59, 287–292.

460 Mombo, L.-E., Ntouni, F., Bisseye, C., Ossari, S., Lu, C. Y., Nagel, R. L., &
 461 Krishnamoorthy, R. (2003). Human genetic polymorphisms and asymptomatic
 462 Plasmodium falciparum malaria in Gabonese schoolchildren. *The American Journal*
 463 *of Tropical Medicine and Hygiene*, 68, 186–190.

464 Nmorsi, O. P., Isaac, C., Ukwandu, N. C. ., & Ohaneme, B. . (2010). Pro–and anti–
 465 inflammatory cytokines profiles among Nigerian children infected with Plasmodium
 466 falciparum malaria. *Asian Pacific Journal of Tropical Medicine*, 3(1), 41–44.

467 Noone, C., Parkinson, M., Dowling, D. J., Aldridge, A., Kirwan, P., Molloy, S. F., O'Neill,
 468 S. M. (2013). Plasma cytokines, chemokines and cellular immune responses in pre-
 469 school Nigerian children infected with Plasmodium falciparum. *Malaria Journal*, 12,
 470 5. doi:10.1186/1475-2875-12-5

471 Ozhan, G., Yanar, H., Ertekin, C., & Alpertunga, B. (2010). Polymorphisms in Tumour
 472 Necrosis Factor Alpha (TNFα) Gene in Patients with Acute Pancreatitis. *Mediators*
 473 *of Inflammation*, 2010, 1–6.

474 Perlmann, P., & Troye-Blomberg, M. (2002). Malaria and the immune system in
 475 humans. *Chemical Immunology*, 80, 229–42. Retrieved from
 476 <http://www.ncbi.nlm.nih.gov/pubmed/12058641>

477 Piel, F. B., Howes, R. E., Patil, A. P., Nyangiri, O. A., Gething, P. W., Bhatt, S., Hay, S.
 478 I. (2013). The distribution of haemoglobin C and its prevalence in newborns in
 479 Africa. *Scientific Reports*, 3, 1671. doi:10.1038/srep01671

480 Pociot, F., D'Alfonso, S., Compasso, S., Scorza, R., & Richiardi, P. M. (1995).
 481 Functional analysis of a new polymorphism in the human TNF alpha gene
 482 promoter. *Scand J Immunol*, 42, 501–504.

483 Rockett, K., Clarke, G., Fitzpatrick, K., Hubbart, C., Jeffreys, A., Rowlands, K., ...
 484 Kwiatkowski, D. (2014). Reappraisal of known malaria resistance loci in a large
 485 multicenter study. *Nature Genetics*, 46, 1197–204.

486 Santovito, A., Cervella, P., Schleicherova, D., & Delpero, M. (2012). Genotyping for
 487 cytokine polymorphisms in a Northern Ivory Coast population reveals a high
 488 frequency of the heterozygote genotypes for the TNF- α -308G/A SNP. *International*
 489 *Journal of Immunogenetics*, 39, 291–5. doi:10.1111/j.1744-313X.2012.01086.x

490 Sohail M., Kaul A., Bali P., Raziuddin M., Singh MP., Singh OP., Dash AP., Adak T.
 491 (2008). Alleles -308A and -1031C in the TNF-alpha gene promoter do not increase
 492 the risk but associated with circulating levels of TNF-alpha and clinical features of
 493 vivaxmalaria in Indian patients. *Mol Immunol*. 45:1682-92

495 Toure, O., Konate, S., Sissoko, S., Niangaly, A., Barry, A., Sall, A. H., Doumbo, O.
 496 (2012). Candidate polymorphisms and severe malaria in a Malian population. *PloS*
 497 *One*, 7(9), e43987. doi:10.1371/journal.pone.0043987

498 Wattavidanage, J., Carter, R., Perera, K. L., Munasingha, A., Bandara, S., McGuinness,
 499 D., Wickramasinghe, A R., Alles, H. K., Mendis, K. N., Premawansa, S. (1999).
 500 TNFalpha*2 marks high risk of severe disease during Plasmodium falciparum
 501 malaria and other infections in Sri Lankans. *Clinical and Experimental Immunology*,
 502 115, 350–355. doi:10.1046/j.1365-2249.1999.00804.x

503 Wilson, A. G., Symons, J. A., McDowell, T. L., McDevitt, H. O., & Duff, G. W. (1997).
 504 Effects of a polymorphism in the human tumor necrosis factor a promoter on
 505 transcriptional activation. *Proc. Natl. Acad. Sci. USA*, 94, 3195–3199.

506 WHO. (2010). *Basic malaria microscopy: part I. learner's guide*.
 507 *Basic Malaria Microscopy: Part I*. Retrieved from
 508 http://whqlibdoc.who.int/publications/2010/9789241547826_eng.pdf

509 WHO. (2014). Malaria. In: World Malaria Report. WHO Global Malaria Programme.
 510 www.who.int

511 Zhang, G., Li, Z., Han, Q., Li, N., Zhu, Q., Li, F., ... Liu, Z. (2011). Altered TNF-α and
 512 IFN-γ levels associated with PD1 but not TNFA polymorphisms in patients with
 513 chronic HBV infection. *Infection, Genetics and Evolution*, 11(7), 1624–1630.
 514 doi:10.1016/j.meegid.2011.06.004

515

516