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Review Article

A meta-analysis of TLR4 and TLR9 SNPs implicated in severe malaria

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Abstract

Toll-like receptors (TLRs) are critical mediators of the inflammatory response to malarial infection, and gene polymorphisms affecting TLR function may be partially responsible for inter-individual variation in disease manifestation. However, there are inconsistencies in the associations of common genetic variants of *TLR4* (D299G) and *TLR9* (T-1237C and T-1486C) with malaria outcome. A comprehensive search was conducted to identify relevant and independent *Plasmodium falciparum*-infected case-control studies, and meta-analysis including six studies for each SNP was performed to obtain more precise estimates of the pooled effects of these variants. The results showed significant associations of the -1486C allele with the risk of severe malaria in allele contrast (T vs. C, p = 0.004, OR = 1.26) and homozygous (TT vs. CC, p = 0.03, OR = 1.51) genetic models. There was no association between the D299G or T-1237C variants and uncomplicated or severe malaria using any of the genetic models tested. However, in stratified analysis, -1237C was associated with the risk of severe malaria in Indian adults (TT vs. TC, p = 0.06, OR = 2.13; TT vs. TC+CC, p <0.00001, OR = 2.65), suggesting that our results must be considered preliminary. The robustness of -1486C as a risk factor warrants investigation into its functionality in malaria pathogenesis. Further, the lack of an association with the T-1237C variant was weak, and future studies examining more detailed individual data from different ethnic groups are essential for confirmation of its genetic contribution to malaria.

Keywords: Malaria.Toll like receptors. Meta-analysis. Polymorphisms.

INTRODUCTION

Malaria, one of the most deadly infectious diseases in humans, is caused by five species of *Plasmodium* (*Plasmodium falciparum, Plasmodium vivax, Plasmodium malariae, Plasmodium ovale,* and *Plasmodium knowlesi*) with an estimated 198 million cases and 584,000 deaths in 2013[1]. Of these, infection due to *P. falciparum* is often associated with a life-threatening spectrum of discrete as well as overlapping symptoms of malaria[2], contributing to about 80-90% of the total global malarial mortality. Despite its high incidence, the low mortality rate (1-2%) in malaria-infected patients even after effective clinical management highlights the importance of host factors, in addition to parasite factors and environmental determinants. The host response to malarial infection is immediate and is mediated by pattern recognition receptors of the innate immune system[3,4]. Among these, the Toll-like receptors (TLRs) are well studied in malaria[5-9]and are stimulated by infected red blood cells (RBCs) and/or parasite-derived metabolites, culminating in the release of pro-inflammatory cytokines such as IFN-γ, IL-12, and TNF-α, as well as nitric oxide[8,9], which are important for controlling the acute blood-stage infection. However, an excessive or deregulated inflammatory response may result in severe malaria.

Of the ten TLRs in humans, the most notable are TLR4 and TLR9, whose activation and changes in expression are thought to potentially affect the clinical outcome of malaria[4-7]. While TLR4 may interact with extra-cellular ligands (such as *Pf*GPI; *Plasmodium falciparum* Glycosylphosphatidylinositol) as well as intracellular ligands as it is located both on the cell surface and on endosomes, TLR9 interacts only with intracellular ligands owing to its endosomal localization[8,10-12]. Interestingly, both TLR4 and TLR9 are influenced by hemozoin (Hz) from ruptured schizonts in malaria. More specifically, Hz facilitates the entry of parasite DNA into the host cell and thereby stimulates TLR9[9], whereas, by interacting with host fibrinogen, Hz activates TLR4 in human monocytes[12]. Since both TLR4 and TLR9 are localized to the endosome and stimulated by Hz, they may share a common influence on the clinical manifestation of malaria. However, genetic association studies analyzing variants in TLR4 and TLR9 from different endemic regions have been conflicting.

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The TLR4 gene, located on the long arm of chromosome 9, has three exons spanning up to 10kb. The two most prevalent non-synonymous SNPs in TLR4, D299G (rs4986790) and T399I (rs4986791), have been studied in different malarial endemic regions and are often co-segregating. A recent study suggested that the D299G SNP influenced TLR4 signaling and was thus functionally important[13].The D299G variant has been associated with reduced surface expression of TLR4, altered binding of *Pf*GPI, and varying cytokine responses to lipopolysaccharides (LPS)[14-16]. The *TLR9* gene, located on chromosome 3p21.3, spans up to 5kb and consists of two exons. Although the inhibition of TLR9 activation by its antagonist or deficiency in mice confers protection against cerebral malaria[17,18], the two most common promoter variants of *TLR9*, T-1237C (rs5743836) and T-1486C(rs187084), have a cis-regulatory effect on *TLR9* expression[19], with various consequences for disease outcome or altered cytokine levels in malaria[20].

In spite of the publication of several genetic association studies, no clear consensus on the effect of *TLR4* or *TLR9* variants on the clinical manifestation of malaria has thus far been obtained. This could be attributed to the inclusion of incorrectly defined or partially matched controls, different study designs, relatively small sample sizes, population stratification, or genetic heterogeneity. Therefore, it is essential to perform a comprehensive search and meta-analysis of these studies in order to verify whether these SNPs are associated with the risk or severity of malaria. Meta-analysis is a powerful tool that has been employed to reach a consensus by analyzing data addressing the same research problem in global malarial settings with different ethnic populations. Although in the majority of previous studies, TLR variants have been compared between healthy controls and either severe malaria (SM) or uncomplicated malaria (UM) patients to determine the severity of or susceptibility to malaria, respectively, there have been limited studies comparing variants in severe cases of malaria with *P. falciparum*-infected controls [mild or asymptomatic (reviewed in ref. 2)]. However, in regions hyper-endemic for malaria it is hard to obtain endemic controls for two reasons. First, although an individual may be healthy at the time of blood draw, he/she might have had previous infections with the malaria parasite and suffered from clinical symptoms; in fact, repeated antigen exposure from infective bites might have led to premunition. Second, because of the strategic control of mosquito-human contact (use of insecticides, bed-nets, etc.), individuals who appear to be healthy are not essentially endemic controls because they may not have been exposed to malaria at all; there remains the possibility that they may develop clinical symptoms upon exposure. The screening of mutations in these apparently healthy individuals and the inclusion of their data, therefore, may not truly fulfill the requirements for controls. Therefore, in order to obtain more precise estimates of the pooled effects of TLR variants with increased statistical power, we carried out a meta-analysis combining relevant data from several independent *P. falciparum*-infected case-control studies conducted to evaluate the association between the *TLR4* D299G and *TLR9*T-1237C and T-1486C polymorphisms and the risk of severe malaria.

METHODS

Identification of eligible studies and data extraction

Published articles in the English language as of the middle of October, 2016 were retrieved from literature search databases such as PubMed, Google Scholar, Science Direct, and MEDLINE using the following key words:*TLR4 variants/polymorphism* and *TLR9 variants/polymorphism* combined with *malaria or Plasmodium*. Two authors independently searched for literature and extracted data in duplicate. Discrepancies were adjudicated until consensus was reached after consultation with another author. References in identified studies were also examined to include any additional studies not indexed by the databases. The selection criteria were then applied to all potentially relevant studies. The criteria for inclusion in the meta-analysis were: I) independent *P. falciparum*-infected case-control or cohort studies conducted to evaluate the association of the *TLR4*D299G and/or *TLR9*T-1237C and T-1486C polymorphisms with the risk of severe malaria in order to exclude the possibility of repeated data from two or more studies from the same region, if any; II) sufficient published data on the genotypes or allele frequencies for determining odds ratios (OR) and confidence intervals (CIs); and III) the study was published as a full paper, not as a meeting abstract or review. The exclusion criteria included: I) non-case-control studies; II) studies containing duplicate data; III) studies based on incomplete raw data or with no reported useable data; IV) studies without clearly defined cases and control, and V) case reports, letters, reviews, and editorial articles. For each included study, data such as the first author, year of publication, ethnicity, type of population described (adults,children $\leq$ 14 years, and pregnant women), polymorphisms tested, genotyping methods, number of cases and controls, number of individuals with different genotypes, and evidence of Hardy-Weinberg equilibrium (HWE) were collected.

Statistical analysis

The genotype distributions of included studies under HWE were assessed by χ^2 tests using an online tool (http://www.oege.org/software/hwe-mr-calc.shtml). The OR of each study was considered the point estimate of risk and was calculated along with the 95% CI. The extent of heterogeneity across studies was determined by χ^2 test based on Cochran's Q statistic and the inconsistency index (I^2). A cut-off point of I^2>50% was based on Cochrane reviews and represented substantial heterogeneity across studies. When a significant Q-test ($P < 0.10$) or an I^2>50% indicated heterogeneity across studies, a pooled OR and 95% CI were estimated using the random-effects model; otherwise the fixed-effects model was used, and the statistical significance was determined based on a P-value < 0.05 using the Z-test. All P-values were two-sided. Begg's funnel plots were created to investigate potential publication bias and small study effects. To assess the influence of a study on the stability of the results, sensitivity analysis was performed by excluding one study at a time whose genotype distributions were not in HWE. For all analyses, statistically significant results were determined to be those with a P-value <0.05, except for tests of publication bias and the Q-test, where P <0.1 was used as the significance level. All statistical analyses were performed using RevMan 5.1

RESULTS

A total of 19 studies investigating the association of the *TLR4* D299G and/or *TLR9*T-1237C and T-1486C polymorphisms with the risk of severe malaria were identified for potential inclusion in the meta-analysis. Of these, five studies (two Indian and three African) fulfilled the inclusion criteria after detailed evaluation (**Figure 1** and **Table 1**). Interestingly, in each of these included studies, all three SNPs were investigated for their association with malaria. However, the study by Sawain et al.[21] from Assam, India had two sets of data for two different ethnic groups (Tibeto-Burman and Austro-Asiatic), which were included as two separate studies in the meta-analysis (**Table 2**). In other words, six studies were evaluated for each of the SNPs.

A summary of the meta-analysis results regarding the associations of TLR gene polymorphisms and the risk of severe malaria is shown in **Table 2**. The HWE analysis of genotype distributions showed significant deviations of the studied SNPs from HWE only in the Indian studies. While *TLR4* D299G deviated from HWE in Sawain et al. in both ethnic populations (Tibeto-Burman and Austro-Asiatic), the *TLR9* promoter variants showed deviations in both the Sawain et al.[21] and Kar et al.[2]studies. The African studies did not show any deviation from HWE in the genotype distributions of any of the studied SNPs (**Table 1**). The sample sizes of malaria-infected patients

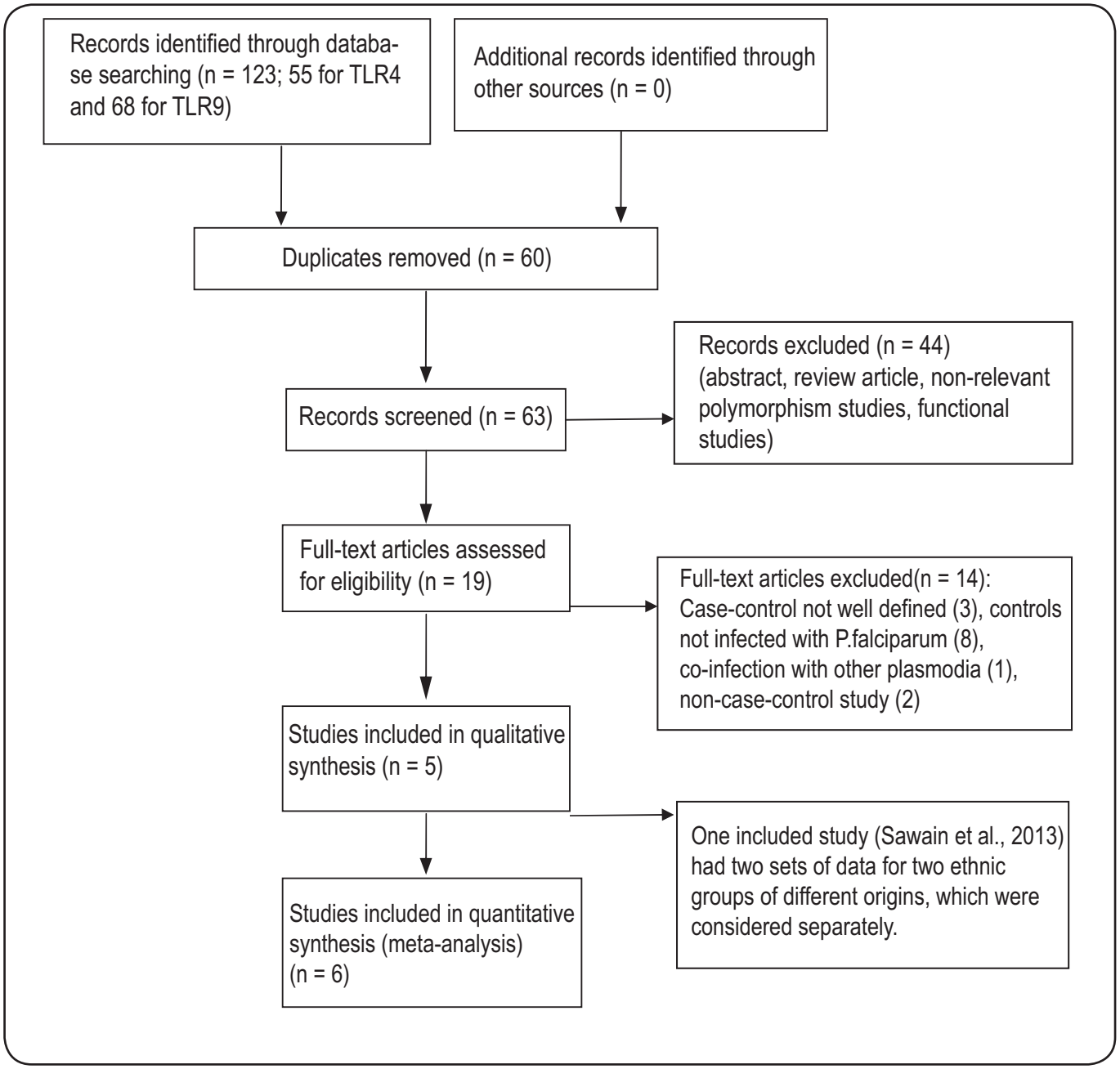

FIGURE 1 - Flow chart for selection of publications included in the meta-analysis. TLR: Toll-like receptor.

TABLE 1

Characteristics of studies included in the meta-analysis.

Author	Age	Control	MAF	D299G			HWE χ^2	MAF	T-1237C			HWE χ^2	MAF	T-1486C			HWE χ^2	Findings	Country
		Cases		DD	DG	GG			TT	TC	CC			TT	TC	CC			
Mockenhaupt et al., [22]	Children	UM	0.12	224	64	2	1.27	0.46	85	144	61	0	0.28	152	114	24	0.16	*TLR4* D299G conferred increased risk of severe malaria compared to healthy controls, *TLR9*promoter variants did not show a clear association with malarial severity	Ghana, Africa
		SM	0.12	220	65	5	0.01	0.42	94	148	48	0.64	0.34	129	126	35	0.24		
Sam-Agudu et al., [20]	Children	UM	0.04	47	5	0	0.13	0.29	24	25	3	1.15	0.25	28	21	3	0.13	-1237CC was more common in CM and associated with high IFNγ. D299G and T-1486C had no effect.	Uganda, Africa
		SM	0.06	57	8	0	0.28	0.4	25	28	12	0.68	0.26	34	27	4	0.2		
Sawian et al.,[21] (A-A)	Adults	UM	0.09	40	5	2	**6.99**	0.27	21	26	0	**6.87**	0.37	18	23	6	0.1	T-1237C was associated with complicated and frequent malaria	India (A-A)
		SM	0.14	24	5	2	**3.8**	0.44	4	30	0	**21.19**	0.26	14	13	1	0.95		
Sawian et al., [21](T-B)	Adults	UM	0.09	38	4	2	**8.91**	0.35	14	30	1	**9.31**	0.46	9	32	6	**6.34**	The -1486T/C genotype was protective against severe malaria	India (T-B)
		SM	0.09	26	4	1	2.13	0.39	7	21	1	**7.63**	0.51	9	11	10	2.12		
Esposito et al., [7]	Children	UM	0.06	481	70	2	0.1	0.37	226	243	84	1.93	0.24	316	201	36	0.28	SM cases were SOD patients. T-1237C was risk factor for susceptibility, not severity.299G was more common in UM, and T-1486C had no effect on susceptibility or disease severity	Burundi, Africa
		SM	0.02	47	2	0	0.02	0.36	19	24	6	0.14	0.27	26	19	4	0.04		
Kar et al., [2]	Adults	UM	0.13	146	49	3	0.24	0.14	160	24	16	**50.33**	0.36	76	101	23	1.49	-1237C and -1486C wererisk factors for SM. -1237CC wasprotective against MODS.	Odisha, India
		SM	0.16	141	52	6	0.2	0.10	159	37	2	0.01	0.45	38	139	21	**34.01**		

T-B: Tibeto-Burman; **A-A:** Austro-Asiatic; **M:** months; **Y:** years; **UM:** uncomplicated malaria; **SM:** severe malaria; **MAF:** minor allele frequency; **CM:** cerebral malaria. **TRL:** Toll-like receptor; **MAF:** minor allele frequency; **HWE:** Hardy-Weinberg equilibrium; **T-B:** Tibeto-Burman; **A-A**: Austro-Asiatic; **M:** months; **Y:** years; **UM:** uncomplicated malaria; **SM:** severe malaria; **SOD**: single organ dysfunction; **MODS**: multi-organ dysfunction.

categorized into UM (controls) and SM (cases) varied for each SNP in the analysis, as shown in **Table 2**.

The findings of the meta-analysis revealed a significant association between the *TLR9*-1486C allele and the risk of severe malaria under both allele contrast (T vs.C, p = 0.004, OR = 1.26, 95% CI = 1.05-2.2) and homozygous (TT vs.CC, p = 0.03, OR = 1.51, 95% CI = 1.11-1.87) genetic models (**Figure 2A** and **B**). Although, the T-1237C variant was not found to be significantly different between UM and SM subjects under any of the genetic models, the TT vs. TC+CC comparison showed a borderline significant association between -1237C and severe malaria (p = 0.08, OR = 1.59, 95% CI = 0.95-2.69; **Table 2**). However, significant heterogeneity between studies (I^2 = 76%) was observed for this comparison. When subgroup analysis was performed, stratification of studies into age groups (adults and children) automatically stratified the subjects by ethnicity [Asian (India) vs. African] because of the non-availability of studies of reciprocal ages in Asia and Africa. In the stratified analysis, while the -1237C allele was significantly associated with the risk of severe malaria in Indian adults (TT vs. TC, p = 0.06, OR = 2.13, 95% CI = 0.97-4.68; TT vs. TC+CC, p <0.00001, OR = 2.65, 95% CI = 1.79-3.91), the -1486C allele was significantly associated in African children (T vs. C, p = 0.03, OR = 1.25, 95% CI = 1.02-1.53; TT vs. CC, p = 0.06, OR = 1.58, 95% CI = 0.98-2.55).

Heterogeneity between studies was observed only for the *TLR9* promoter variants. None of the models for the genotype and allele distributions of the *TLR4*D299G variant, including stratified analysis, were found to be significantly different between UM and SM subjects. We did not find any significant difference in the distributions of the studied SNPs between UM and SM subjects after sensitivity analysis (data not shown), indicating that the results of the meta-analysis were reliable and stable. The presence of publication bias and small study effects were assessed by funnel plots and Begg's tests, and no significant biases were observed.

DISCUSSION

The Toll-like receptors (TLRs) are critical mediators of the inflammatory response to malarial infection[3,4] and in endemic areas of the disease, gene polymorphisms affecting TLR function may be partially responsible for inter-individual variation in disease manifestation. However, there have been inconsistencies in the results of association studies of the common genetic variants of *TLR4* (D299G) and *TLR9* (T-1237C and T-1486C) on the clinical outcomes of malaria. In order to verify the genetic contributions of these variants to malaria with more precision, we performed a comprehensive search and pooled the results of independent studies for meta-analysis. Our meta-analysis results for the *TLR4*D299G variant were consistent with those of previous studies on diverse ethnicities from different malarial endemic regions[2,20,22], with no difference in the genotype or allele frequency distributions of the D299G polymorphism between UM and SM subjects. Although the contribution of the 299G minor allele has been controversial in children[7,20,22,23], adult studies show no association[2,21,24] with the exception of maternal anemia in pregnant women[25]. This could be due to a lack of adequate studies, ethnic differences, or the fact that the effect of 299G may vary with age groups. However, we did not find any association between this polymorphism and severe malaria, even after stratifying the meta-analysis by age and ethnicity, though a substantial amount of heterogeneity was observed in children (data not shown). Moreover, many genetic association studies of the *TLR4* D299G variant show no evidence for its association with malaria, either in terms of malarial susceptibility or resistance[26-28]. These observations strongly suggest that the *TLR4* D299G polymorphism is a non-biomarker for malaria.

Although, the -1237C allele of *TLR9* has been associated with disease susceptibility[7] and risk of severe malaria[2,20,21], our meta-analysis results did not reveal a significant association.

TABLE 2

Association of TLR SNPs (*TLR4*D299G, *TLR9*T-1237C, and *TLR9* T-1486C) with risk of severe malaria in meta-analysis.

SNPs	Genetic Model	Sample Size		Model	Heterogeneity		Association		Overall effect	
		Cases	Controls		I^2 (%)	P-value	OR	95% CI	Z-value	P-value
*TLR9*T-1237C	TT vs. CC	377	695	Random	65	0.02	0.81	0.34–1.95	0.47	0.64
	TT vs. TC	596	1022	Random	50	0.08	1.34	0.91–1.99	1.48	0.14
	TT vs. TC+CC	665	1187	Random	76	0.001	1.59	0.95–2.69	1.75	0.08
	TT+TC vs. CC	665	1187	Random	66	0.02	0.79	0.34–1.83	0.56	0.58
	T vs. C	1330	2374	Random	57	0.04	1.06	0.80–1.41	0.44	0.66
*TLR9*T-1486C	TT vs. CC	325	697	Fixed	0	0.6	1.51	1.05–2.2	2.19	0.03
	TT vs. TC	585	1091	Random	70	0.005	1.17	0.73–1.87	0.66	0.51
	TT vs. TC+CC	660	1189	Random	63	0.02	1.23	0.82–1.85	1	0.32
	TT+TC vs. CC	660	1189	Fixed	23	0.26	1.26	0.90–1.78	1.34	0.18
	T vs. C	1320	2378	Fixed	6	0.38	1.26	1.08–1.48	2.9	0.004
*TLR4*D299G	DD vs. GG	529	987	Fixed	0	0.95	1.86	0.81–4.27	1.46	0.14
	DD vs. DG	651	1173	Fixed	0	0.56	1.02	0.78–1.34	0.17	0.87
	DD vs. DG+GG	665	1184	Fixed	0	0.53	1.06	0.82–1.38	0.46	0.64
	DD+DG vs. GG	665	1184	Fixed	0	0.94	1.81	0.79–4.16	1.41	0.16
	D vs. G	1330	2368	Fixed	0	0.52	1.10	0.87–1.38	0.77	0.44

OR: odds ratio; **CI:** confidence interval; **I^2:** inconsistency index; **TLR:** toll like receptor.

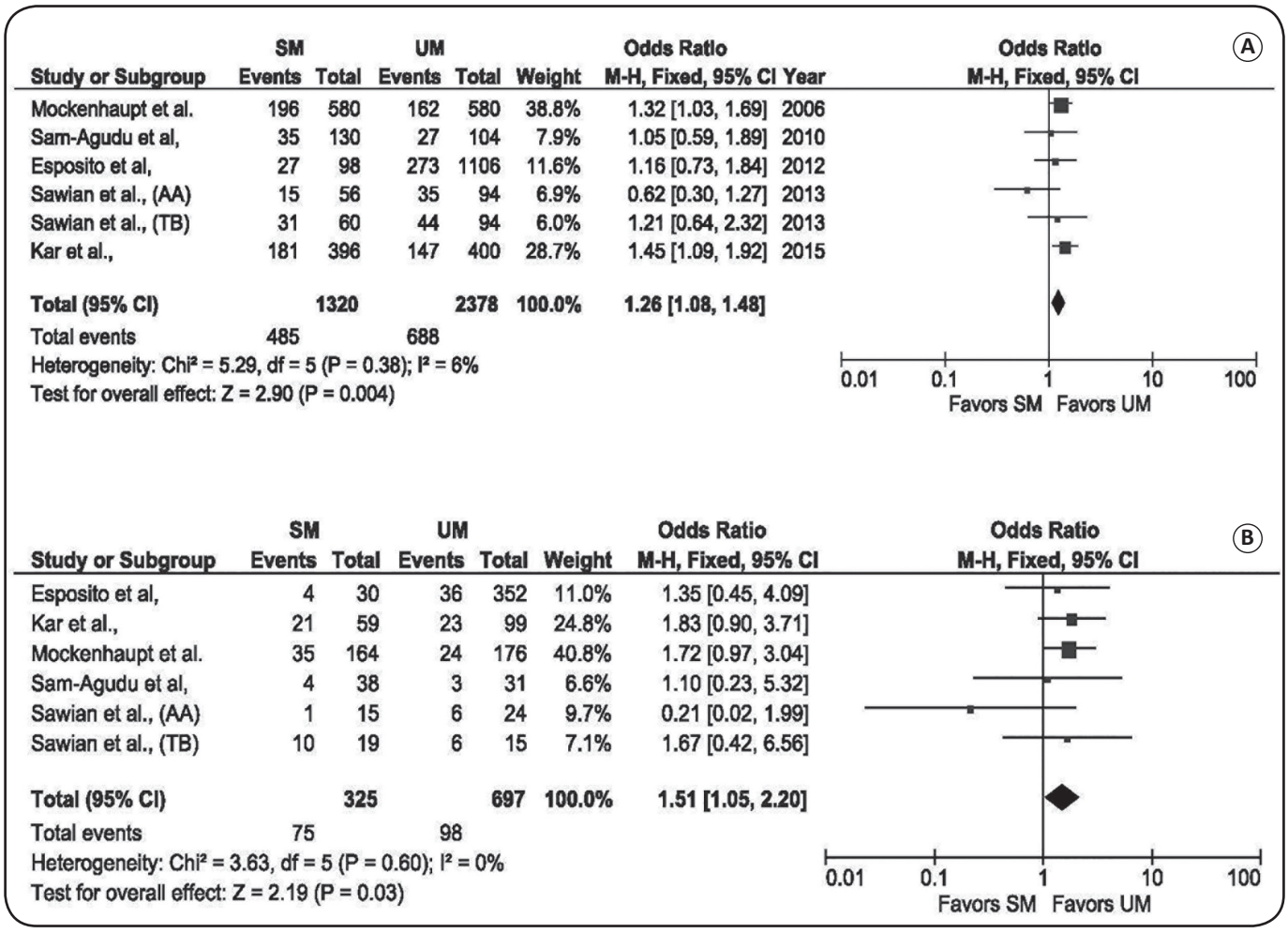

FIGURE 2 - Forest plots for the association of *TLR9*T-1486C with the risk of severe *Plasmodium falciparum* malaria. (A). Result under allele contrast (T vs. C) model. (B). Result under homozygous genotype model (TT vs. CC). A fixed-effects model was used. The squares and horizontal lines represent the study-specific OR and 95% CI, respectively. The diamond represents the pooled OR and 95%CI. **TLR:** Toll-like receptor; **SM:** severe malaria; **UM:** uncomplicated malaria; **95% CI**: confidence interval 95%;.

However, the significant heterogeneity between studies of the TT vs. TC+CC comparison and its borderline association with the risk of severe malaria suggests that the estimate of the contribution of the -1237C allele may have been affected. Further, the stratified meta-analysis result of a significant association between the -1237C allele and severe malaria in Indian adults indicates that the estimate of the potential contribution of the -1237C allele to malaria could have been affected by confounding biases like population stratification or a varying age effect. In addition, our recent observation of opposing associations of the -1237C allele with different sub-clinical severe malarial phenotypes such as single organ dysfunction (SOD) vs. multi-organ dysfunction (MODS)[2] suggests that phenotype misclassification in malaria could also lead to a reduction in the power to detect the contribution of this variant. This may explain the lack of observed associations between the T-1237C variant and susceptibility or resistance to malaria[22,27,29,30], in addition to other factors. Therefore, future studies examining large sample sizes of malaria-infected patients categorized into required phenotype classes from different ethnic populations and belonging to different age groups are eagerly awaited for confirmation.

Similarly, no clear consensus has been obtained for the involvement of the T-1486C polymorphism in malaria. Although some studies have reported no association between T-1486C and malaria susceptibility or severity[7,20,22,24,27,30], we observed that the -1486C allele was a risk factor for severe malaria and high parasite load, which is in line with previous findings[21,24]. Moreover, -1486C has been associated with an increased risk of low birth weight in term infants born to *P. falciparum*-infected pregnant women[25].Consistent with these findings, the meta-analysis of the T-1486C variant revealed a significant association with SM under the allele contrast (T vs. C) and homozygous (TT vs. CC) genetic models. Despite the fact that the stratified analysis did not detect a statistically significant association in Indian adults, its association in African children, both in T vs. C and TT vs. CC models, suggests that the findings are robust, at least in children. Further, as with

the -1237C variant, -1486C could also be affected by population stratification or varying age effects. In addition, the existence of linkage disequilibrium between *TLR9* promoter SNPs and cis-acting regulatory variants influencing *TLR9* expression[19] suggests an influence of the host genetic makeup on malaria risk and could lead to discrepancies in the association observed in different studies. Interestingly, in the absence of considerable publication bias, the deviation of the *TLR9* promoter variants from HWE, which was found to be associated with the risk of severe malaria in meta-analysis, suggests the operation of natural selection processes at these loci. Although all three studied SNPs showed deviations from HWE in Sawain et al.[21] (which could be due to the small sample size), the distribution of *TLR4* D299G at par with HWE in all other studies was apparent because of its neutral role as observed in meta-analysis.

To the best of our knowledge, this is the first meta-analysis assessing the associations of the *TLR4*D299G and *TLR9*T-1237C and T-1486C polymorphisms with risk of severe malaria. However, there are certain limitations that should be noted while interpreting these results. First, the eligible studies in the present meta-analysis were mainly from Asian adults and African children, which may prevent us from obtaining robust estimates for the associations. Data from other populations were limited or publication constraints precluded their inclusion in the meta-analysis, and there were no studies with reciprocal ages of subjects in Asia and Africa. Second, only a few studies were included in this analysis, all with relatively small sample sizes, and there was no categorization of the severe malaria group into sub-clinical groups, which might have limited the statistical power to reveal significant associations. Third, the genotyping methods were different among studies, which might lead to heterogeneity and hence may have affected the accuracy of the results. Fourth, a linkage disequilibrium analysis could not be performed because of insufficient individual information on genotypes, which makes it difficult to speculate on the combined interactions of the studied variants.

Despite these limitations, the strength of the present study lay in the fact that the analysis was based on *P. falciparum*-infected malarial samples belonging to SM and UM groups, which avoids any errors in the selection of healthy controls. Further, most of the associations were observed in fixed-effects models, indicating that the influence of heterogeneity between studies on the power of the meta-analysis was low. In cases where significant heterogeneity was observed among studies, we tried to explore the source of the heterogeneity by examining factors such as age group and/or ethnicity. In these cases, a random-effects model was used to derive the overall effect. Of note, the findings of the present study shed light on the possible association of *TLR9* promoter variants with the risk of severe malaria. However, our meta-analysis results must be considered preliminary and interpreted with caution in the context of its limitations. Consequently, future studies with large sample sizes from global malarial settings and with well-defined cases and controls should be performed to verify the present findings.

Conflicts of interest

The authors declare that there is no conflict of interest.

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