Heliyon 8 (2022) e09318

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon

Research article

Association of toll-like receptors in malaria susceptibility and immunopathogenesis: A meta-analysis

Aína Danaisa Ramirez Ramirez [a,1], Myrela Conceição Santos de Jesus [a,b,1], Júlia Rossit [a,1], Nathália Faria Reis [a], Marcelo Cerilo Santos-Filho [a], Adriana Pittella Sudré [c], Joseli de Oliveira-Ferreira [d], Andrea Regina de Souza Baptista [a], Luciane Moreno Storti-Melo [b], Ricardo Luiz Dantas Machado [a,*]

[a] *Center for Microorganisms' Investigation, Department of Microbiology and Parasitology, Biomedical Institute, Fluminense Federal University, Niterói, Rio de Janeiro, Brazil*
[b] *Laboratory of Molecular Genetics and Biotechnology, Department of Biology, Center for Biological and Health Sciences, Federal University of Sergipe, São Cristóvão, Sergipe, Brazil*
[c] *Laboratory of Parasites Molecular Biology, Department of Microbiology and Parasitology, Biomedical Institute, Fluminense Federal University, Niterói, Rio de Janeiro, Brazil*
[d] *Laboratory of Immunoparasitology, FIOCRUZ, Rio de Janeiro, Rio de Janeiro, Brazil*

ARTICLE INFO

Keywords:
Malaria
Plasmodium falciparum
Plasmodium vivax
Polymorphism
TLR
Toll-like receptors

ABSTRACT

Toll-like receptors (TLRs) play a key role in the induced immune response in malaria. Although the potential roles of TLRs have been described, it is necessary to elucidate which of these receptors may actually have an impact on the immunopathogenesis of the disease. This article performed a meta-analysis adhered to the PRISMA statement on TLRs studied in malaria by *Plasmodium falciparum* and *Plasmodium vivax* and its impact on susceptibility and pathogenesis during malaria. A search of the literature was undertaken in PubMed, LILACS and SciELO published until June 30th, 2020. The risk of bias was calculated using the Joanna Briggs Institute's Critical Review Checklist. Later, based on the inclusion and/or exclusion criteria, 17 out of 296 articles were harvested for this systematic review, the meta-analysis included studies incorporating 6,747 cases and 8,983 controls. The results showed that only TLR1, TLR9 and TLR4 receptors were associated with parasitemia, TLR2 and TLR6 were related with severity and none TLR was correlated with susceptibility. The data described here should be taken with caution, since the current evidence is limited and inconsistent. More studies are needed given that the results may change depending on the region and genetic background of the populations.

1. Introduction

Despite advances in decreasing malaria morbidity and mortality in recent years, the disease remains a major public health issue with more than 229 million cases and 409,000 deaths occurring in 2019 alone [1]. Currently, there are five species of *Plasmodium* spp. capable of infecting humans, with *P. falciparum* being the main etiological agent in the African continent and responsible for the majority of severe cases and malaria-related deaths [2]. Outside the African continent, *P. vivax* is the most widely distributed species [3], being mainly related to cases of morbidity, and the most responsible for cases of malaria relapses due to reactivation of the latent form of *P. vivax,* called hypnozoites, in the liver [4].

Plasmodium parasites are initially recognized by components of the host's innate response, with its early identification mediated by pattern recognition receptors (PRRs) which pinpoint pathogen-associated molecular patterns (PAMPs). One of the most important PRRs are Toll-like receptors (TLRs). TLRs are a subgroup of transmembrane receptors present in epithelial, endothelial, natural killer and dendritic cells, and in monocytes, macrophages, and neutrophils [5, 6]. During *P. falciparum* malaria, the activation of the response is initiated by the recognition of the specific components of the glycosylphosphatidylinositols (GPIs), as well as parasite DNA and RNA, which are considered to be important pathogenic factors. This recognition happens through the heterodimers TLR2/1 and TLR2/6 and their activation leads to signaling cascades that, among other actions, induce the release of cytokines [7].

* Corresponding author.
E-mail address: ricardomachado@id.uff.br (R.L.D. Machado).
[1] These authors have contributed equally.

https://doi.org/10.1016/j.heliyon.2022.e09318
Received 24 November 2021; Received in revised form 15 February 2022; Accepted 20 April 2022

Since the first report of a Toll protein in the fruit fly *Drosophila melanogaster* [8], 10 TLRs have been identified in humans (TLR1–TLR10). TLRs are either expressed extracellularly on the cell surface (TLR1, 2, 4, 5, 6, and 10) where they can recognize components of the external membrane of pathogens such as proteins, lipids and carbohydrates; or intracellularly on the endosomal membrane (TLR3, TLR7, TLR8 and TLR9) where they recognize pathogen genetic material, such as viral nucleic acids [9]. The extracellular forms TLR2 and TLR4 are also expressed intracellularly in dendritic cells, epithelial and endothelial cells [10].

In the early stage of the disease, the release of pro-inflammatory cytokines is essential for the elimination of the parasite [11]. However, the most severe forms of malaria are also caused by an uncontrolled and exacerbated inflammatory response, amongst other factors [5, 11]. Although studies of the association between genetic variations caused by single-nucleotide polymorphisms (SNPs) and disease outcomes have revealed potential roles for TLR signaling pathways in human malaria [5, 6], there is no clear consensus on which TLRs impact the immunopathogenesis of the disease, or on the impact of SNPs on different TLRs in respect of susceptibility to malaria or the disease's pathogenesis, as evidenced in other infectious diseases [12, 13]. This article, therefore, perform a meta-analysis on previously studied TLRs associated with *P. falciparum* and *P. vivax* malaria and their impact on susceptibility and pathogenesis of the disease.

2. Material and methods

2.1. Search strategy

A systematic search was conducted following the Preferred Reporting Items for Meta-Analyses (PRISMA) statement [14], to identify relevant studies related to Toll-like receptor polymorphisms and human malaria infection by *Plasmodium falciparum* and/or *Plasmodium vivax* from distinct continents. The search for articles was undertaken in the PubMed, LILACS (Latin America and Caribbean Health Sciences Literature) and SciELO (Scientific Electronic Library Online) databases for articles published up to June 30th, 2020. The research question was structured in the PICO format, where P = Patients with malaria; I = types of Toll-like receptors; C = Patients without malaria and O = Disease severity, high parasitemia and susceptibility to malaria. The following search terms were used: ("malaria" AND "Toll-like receptors" AND "*Plasmodium vivax")*; ("malaria" AND "Toll-like receptors" AND "*Plasmodium falciparum*"); ("malaria" AND "Toll-like receptors" AND "Polymorphism" AND "*Plasmodium vivax*") and ("malaria" AND "Toll-like receptors" AND "polymorphism" AND "*Plasmodium falciparum*").

2.2. Inclusion criteria

The Meta-Analyses was performed by searching studies reporting data on Toll-like receptors in human malaria patients. Studies were selected if they met the following criteria: (1) Articles published in journals peer reviewed with a description of the sample strategy and study design; (2) Studies that included cases of patients infected with *P. falciparum* and/or *P. vivax* species; (3) Surveys performed containing details of the laboratory methods and a description of the parasite species, Toll-like receptors and nucleotide sequence alterations (single nucleotide polymorphism or mutation); (4) Demographic population data (children and/or adults, continent/country of residence); (5) Studies were included regardless of the date of publication up to June 30th 2020. Studies that measured cytokine serological levels were also considered. This review included articles available in the selected databases, harvested between June 8, 2020 to June 30, 2020, written either in English, Portuguese or Spanish and that met the above criteria.

2.3. Exclusion criteria

The present work did not consider case reports, experimental studies using animal models or research on pregnant women, as well as systematic reviews. Other non-representative groups were also excluded, such as human patients infected or co-infected with other species than *Plasmodium falciparum* or *Plasmodium vivax* or even by other groups of infectious agents. Also were excluded articles that did not have clearly defined case and control groups, as well as studies that did not have elucidated outcomes.

2.4. Risk of bias

The risk of bias was assessed in each paper by three reviewers using the Joanna Briggs Institute's Critical Review Checklist for case-control and cohort studies [15]. Studies were characterized according to the following risk of bias: "high" when the study reached up to 50% score "yes"; "moderate" when the study reached between 50% and 70% score "yes"; and "low" when the study reached more than 70% score "yes". Only papers with low scores were included.

2.5. Data extraction

Data from each of the included studies were extracted independently by two of the authors, duplicate publications among different databases were excluded. The following information was collected from each eligible study: (1) Study technical features (type and year of publication); (2) Type of Toll-like receptor; (3) the *Plasmodium* species being studied; (4) Characteristics of the population (age groups analyzed, number of participants and continent/country of residence); (5) Malaria susceptibility, parasitemia and severity.

2.6. Statistical analysis

The analysis was performed with Review Manager software Version 5.4 [16], using a random effect model for the correlation between TLRs and severity, parasitemia and susceptibility during malaria. The results were presented according to the odds ratio (OR) with confidence intervals (CI) of 95%. To verify the existence of heterogeneity between the studies, the Cochran Q test and the Higgins and Thompson I^2 statistic were used. In order to explore the heterogeneity, subgroup analysis was performed by type of TLRs. All meta-analysis data were summarized in Forest plot graphs.

3. Results

3.1. General description of the included studies

A total of 296 articles were retrieved from PubMed (n = 168), LILACS (n = 126) and SciELO (n = 2) using the search strategy followed in this study. Of these, 144 were duplicates and removed, and the remaining 152 articles were screened by title and abstract. One hundred and twenty-five further studies were excluded since they did not meet the inclusion or/and exclusion criteria. Twenty-seven articles remained and full texts were thoroughly analyzed. Of these, ten were excluded for not fitting the inclusion criteria, two because they did not compare the TLR with any of the guiding threads of this review (severity, parasitemia and/or susceptibility) and one article was excluded because it also has cases of *P. malariae*, the other seven were excluded because they did not provide sufficient data for meta-analysis. Finally, seventeen articles were included, and the research papers were classified according to the parasite species: nineteen studies investigated patients infected with *P. falciparum,* two with *P. vivax* and three presented infections by both species.

The *P. falciparum studies* were conducted in Africa (n = 5), Asia (n = 6), South America (n = 1) and Oceania continents (n = 1). Just one *P. vivax* study was carried out in South America, while the studies that had both species were conducted in Asia (n = 2) and South America (n = 1) (Figure 1). The articles were published between 2006 and 2018, and

the year with the most selected articles was 2016 (n = 4) (Additional file 1).

Regarding the age of the population in each study, 8 articles included adults only, 7 included children only and 2 had individuals of all ages. Among the TLRs investigated in respect of malaria TLR1, TLR2, TLR4, TLR6 and TLR9 were reported in studies with *P. falciparum* infections, TLR1, TLR4, TLR5, TLR6 and TLR9 in studies with *P. vivax* and TLR2, TLR4 with both species (Additional file 1).

The present meta-analysis incorporating 6,747 cases and 8,983 controls, with 2,097 cases and 1,905 controls included in the susceptibility meta-analysis, 6,555 cases and 5,748 controls in the parasitemia meta-analysis, and 9,343 cases and 9,195 controls in the severity meta-analysis (Figures 2, 3, and 4).

The results regarding the importance of the participation of each TLR in the different stages of *Plasmodium* infection are presented according to disease susceptibility, parasitemia and malaria severity.

3.2. Effect of TLRs on malaria susceptibility

3.2.1. Susceptibility

Nine studies were included in the meta-analysis of this aspect, involving 7,536 cases and 12,753 controls. Heterogeneity was identified by I-square statistic with $I^2 = 98\%$, so a random effect model was used. No significant association between any TLRs studied and susceptibility to malaria was observed in the random effect model (OR: 1.29, 95% CI: 0.73–2.28, p = 0.38, $I^2 = 98\%$) during the meta-analysis (Figure 2).

3.3. Effect of TLRs on malaria severity

3.3.1. Malaria severity

Eleven studies were included in the meta-analysis of this aspect, involving 9,343 cases and 9,195 controls. Heterogeneity was identified by I-square statistic with $I^2 = 98\%$, so a random effect model was also used.

During the overall meta-analysis, no significant association between any TLRs studied and the severity was observed in the random effect model (OR: 1.27, 95% CI: 0.66–2.47, $I^2 = 98\%$) during the overall meta-analysis. However, in the subgroup analysis by each TLR included, TLR6 (OR: 0.62, 95% CI: 0.47–0.83, p = 0.47, $I^2 = 29\%$) increased severe malaria and TLR2 (OR: 12.94, 95% CI: 1.04–160.75, $I^2 = 96\%$) was protective against severe malaria (Figure 3).

3.4. Effect of TLRs on parasitemia

3.4.1. Parasitemia

Six studies were included in the meta-analysis of this aspect, involving 6,555 cases and 5,748 controls. Heterogeneity was identified by I-square statistic with $I^2 = 96\%$, so a random effect model was also used.

No significant association between any TLRs studied and the parasitemia was observed in the random effect model (OR: 0.92, 95% CI: 0.53–1.60, $I^2 = 96\%$) during the overall meta-analysis. However, in the subgroup analysis by each TLR included, TLR1 (OR: 0.45, 95% CI: 0.22–0.91, $I^2 = 92\%$) and TLR9 (OR: 0.32, 95% CI: 0.11–0.95, $I^2 = 97\%$) promoted high parasitemia and TLR4 (OR: 3.97, 95% CI: 1.02–15.47, p = 0.77, $I^2 = 96\%$) was protective against high parasitemia (Figure 4).

4. Discussion

In this study, we performed a meta-analysis to assess the association between TLRs studied and three important aspects of malaria: susceptibility, parasitemia and severity. Susceptibility comprises different genetic, immunological, and physiological factors that interfere in the parasite-host interaction and may prevent the implantation of the Plasmodium in the host [34, 35]. Meanwhile, severity in malaria corresponds to the variety of severe clinical conditions that are caused as a result of the disease. These include central nervous system disorders, severe anemia, renal failure, pulmonary dysfunction, shock, disseminated intravascular coagulation, hypoglycemia, metabolic acidosis, and liver dysfunction [36, 37]. This meta-analysis revealed an association between TLR1 and TLR9 receptors with higher parasitemia, while TLR4 was protective in respect of high parasitemia. Concerning severity, TLR6 favored severe malaria while TLR2 was protective. No TLR analyzed was associated with susceptibility during this meta-analysis.

Toll-like receptors are important molecules for pathogen recognition in the early immune response and can detect *Plasmodium* ligands [7, 8,

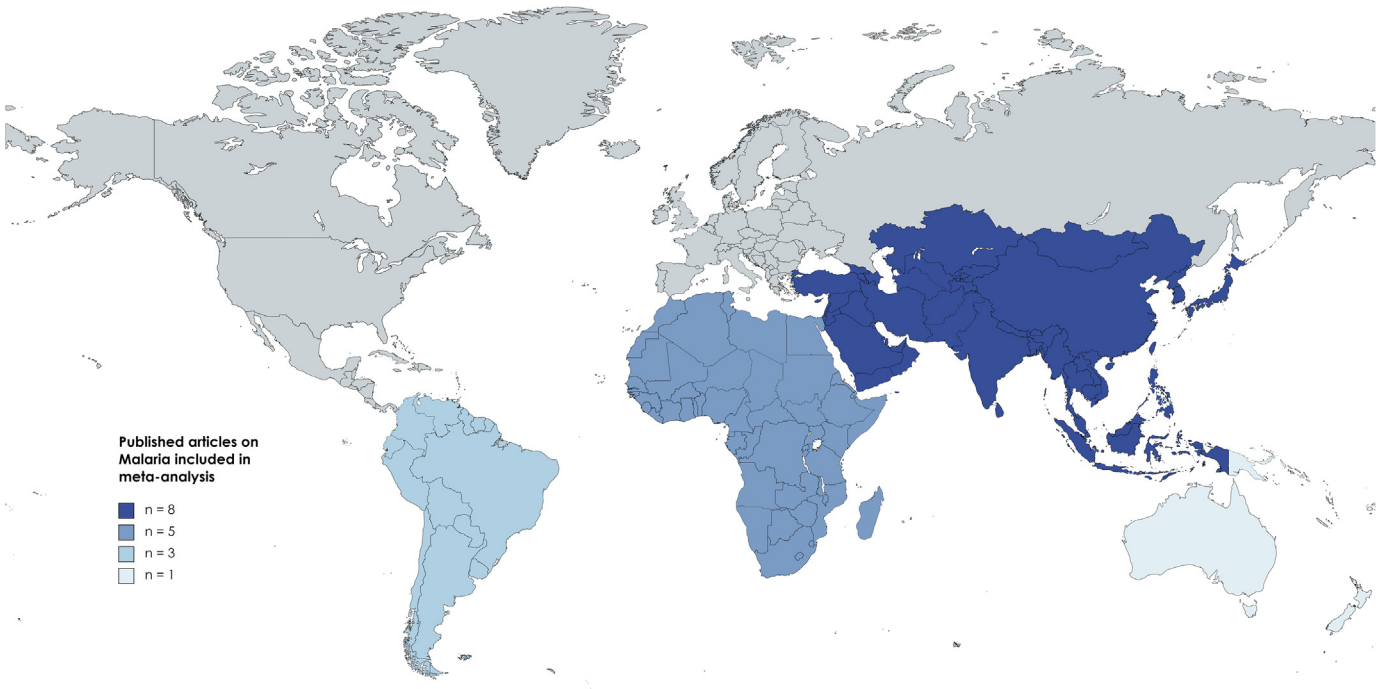

Figure 1. Map of the studies included in the meta-analysis. The darker shades of blue are the continents with the highest number of included studies.

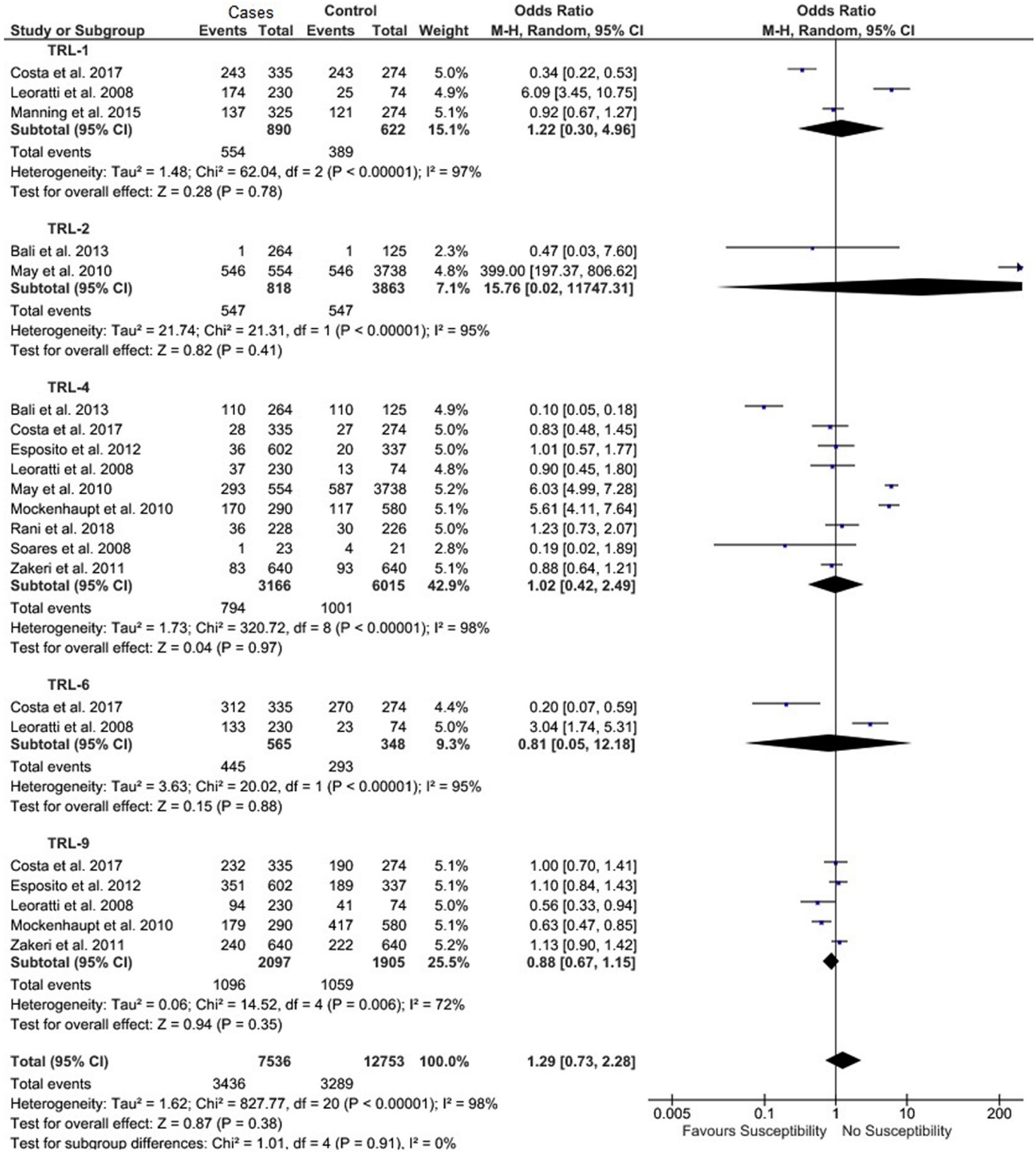

Figure 2. Forest plot for TLRs and malaria susceptibility.

9]. Nevertheless, this study highlighted the limited number of studies that have focused on understanding the role of these receptors in the susceptibility and clinical course of malaria. A total of 17 studies were included with most of the studies being published between 2006 and 2018. The year with the highest number of publications was 2012 (n = 3) while no studies were published in the last two years included: 2019 and 2020. In addition to the low number of articles, we also found that while many studies focused on investigating the function of the adaptive response during malaria [38, 39], few focused on the innate response. Increasing evidence suggests that the TLR family plays an important role in innate immunity, being responsible for the initial recognition of the pathogen, and it is essential in the connection between innate and adaptive immunity [6, 40].

As expected, most articles were with patients infected with *P. faciparum* (n = 13, 76.5%). Of these thirteen studies, Asia (n = 6, 46.1%) and Africa (n = 5, 38.4%) were the continents with the highest

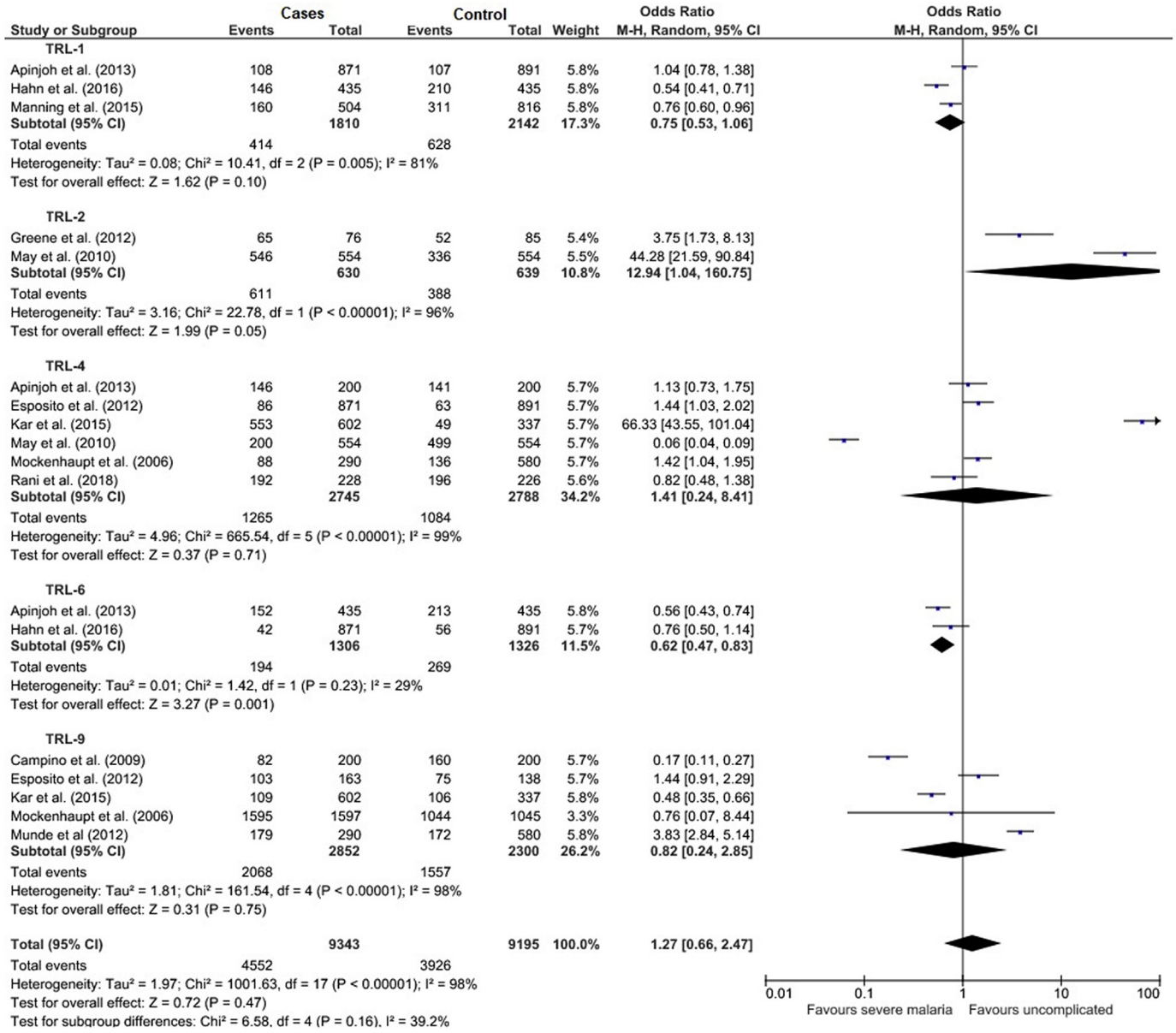

Figure 3. Forest plot for TLRs and malaria severity.

frequency. This occurred because death and severe forms of malaria mainly involve *P. falciparum* [1, 4], and Africa and Asia have the largest burden of malaria cases in the world [1, 3].

The scenario changes drastically with respect to *P. vivax*. The low number of articles included in our search is alarming (n = 1, 5.8%) in view of the role of TLRs in *P. vivax* malaria. Although not often related to deaths, *P. vivax* is the most widely distributed *Plasmodium* species, being responsible for many cases of morbidity [1, 4], and more recently also being described in cases of severe malaria [41, 42]. This species also has epidemiological importance due to the characteristics of the parasite's biology that makes it difficult to interrupt the transmission cycle. Among these characteristics are the early production of gametocytes before the onset of symptoms [43, 44], the existence of latent forms of the parasite in the liver (hypnozoites) [5, 45], a greater number of asymptomatic infections [46, 47] and the immune escape mechanism in the spleen [48].

The fact that only one study was carried out with *P. vivax* highlighted how little attention has been paid to this species. This research was performed by one group in the state of Amazonas, Brazil, who were able to evaluate several TLRs (TLR1, TLR4, TLR5, TLR6 and TLR9) [17].

There were some contradictory results regarding the role of the Toll-like receptor polymorphisms in all three aspects considered in this study. These are presented further in the following section:

4.1. Susceptibility

- TLR1 and TLR6, have conflicting results. Two studies that have investigated the influence of these TLRs with susceptibility found statistical associations, but with opposite results (Figure 2). While one reported that it favors susceptibility [17] the other showed a protective effect of these receptors [32]. Both studies were conducted with adults from South America (Brazil); however, one was conducted with individuals infected with *P. vivax* [17] and the other with *P. falciparum* [32]
- TLR2: the meta-analysis results showed that most studies that evaluated this TLR found that it does not influence susceptibility (Figure 2); however, a study conducted in Africa (Ghana) which included people of all ages infected with *P. falciparum* found that this TLR has a protective role in respect of malaria susceptibility [29].
- TLR4: three studies were significant in our analysis (Figure 2). Of these, two identified a protective action of the receptor in respect of

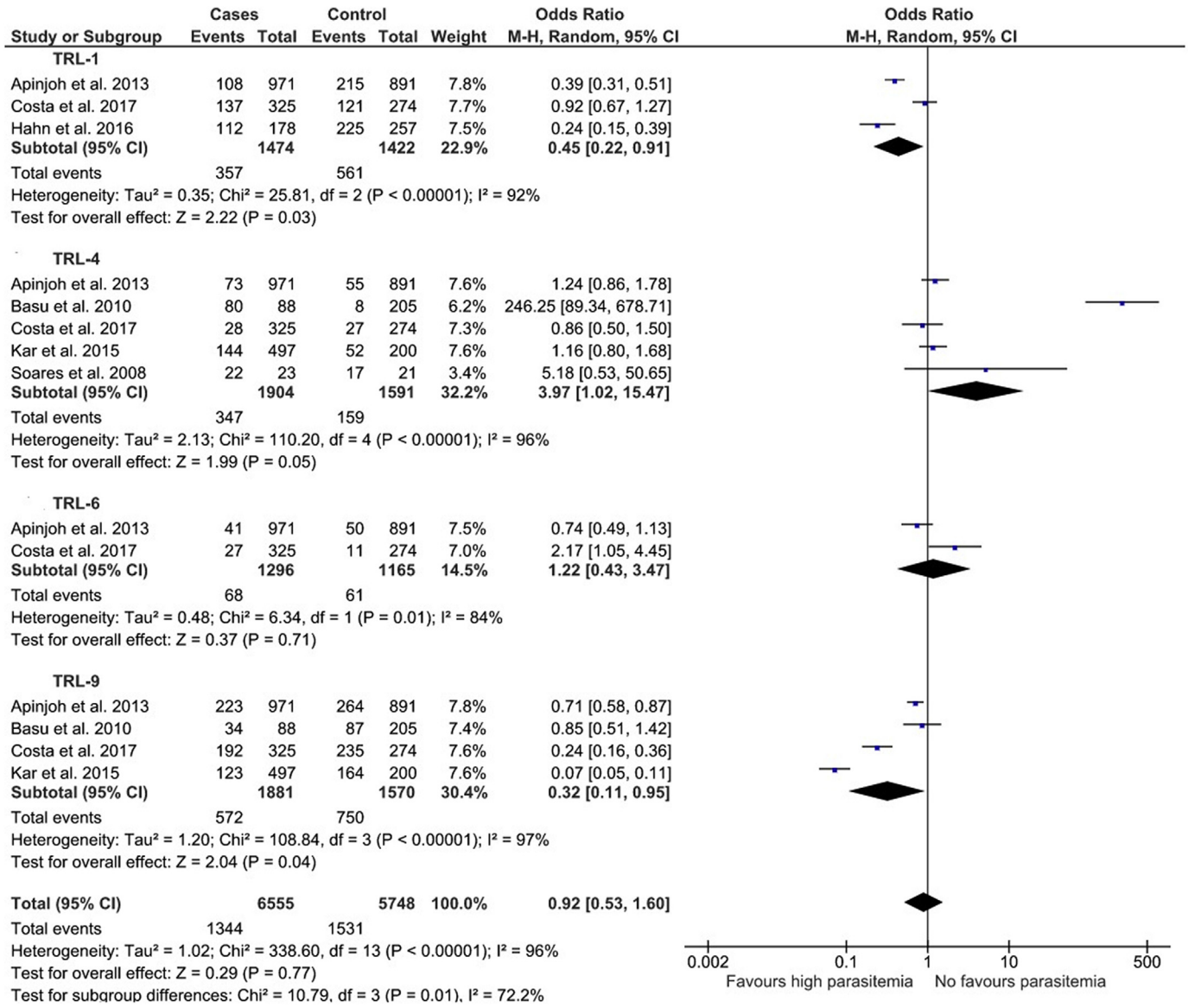

Figure 4. Forest plot for TLRs and parasitemia.

P. falciparum malaria in children and adults from in Africa (Ghana) [29, 33], while the other found that it increased the occurrence of infection by *P. vivax* and *P. falciparum* in individuals of all ages in Asia (India) [22].

- Despite the identification of some individual associations in studies, our meta-analysis found no significance in any of the TLRs analyzed here with respect to susceptibility to malaria by both species (Figure 2). Therefore, the positive results highlighted here cannot be extrapolated to conditions other than those used in the studies.

4.2. Severity

- TLR1: Most studies reported that it does not influence severity (Figure 3). There was only one study that obtained a significant association, showing that this receptor increased disease severity [20]. This study included adults and adolescents from Asia (Myanmar) infected with *P. falciparum*.
- TLR2: Two studies identified a protective action of this receptor against the severe forms of malaria [26, 29] (Figure 3). Both surveys were conducted in Africa (Ghana and Ugandan) and included individuals infected with *P. falciparum*.
- TLR4: Four studies were significant, all of them with *P. falciparum* (Figure 3). Of these, one study conducted with African patients (Ghana) of all ages showed that this receptor increased disease severity [29]. In contrast, three studies showed a protective role for this TLR. Two of them with children from Africa (Burundi and Ghana) [25, 33] and the other with Asian adults (India) [21].
- TLR6: One study found that it increased the appearance of severe forms of malaria in children from Africa (Cameroon) infected with *P. falciparum* [23] (Figure 3).
- TLR9: Two articles reported a positive correlation between this receptor and the development of severe forms of malaria (Figure 3). One was carried out with adults from Asia (India) [21] and the other with children from Africa (Malawi and Gambia) [30].
- Regardless of these individual results, the meta-analysis only indicated the influence of two TLRs with malaria severity: TLR2 protecting from severe forms and TLR6 favoring severity (Figure 3).

4.3. Parasitemia

- TLR1 and TLR9: A study in west-central Africa reported an association between these receptors and greater parasitemia among children [23]. However, a specific genotype of TLR-9 showed an association

with greater parasitic load in two endemic areas in the state of Amazonas, but TLR-1 showed no relation regarding the same parameter in this population [17]. Similar findings were described for TLR-9 in a study from Kolkata, India [28]. On the other hand, in Odisha, a city in the eastern region of India, a negative association with a homozygous genotype of the same SNP was reported [21]. Despite being spread in different receptors, both of these components of the innate immunity were positively linked with high parasitemia (Figure 4).

- TLR-4: The meta-analysis showed a negative association with this receptor and parasitemia, suggesting a protective effect, despite the heterogeneous populations studied (Figure 4). However, it is important to point out that in individual analysis only one research conducted with adults from Asia (India) presented this association [28].
- TLR6: the role of this receptor remains unclear. The overall meta-analysis indicated no influence with high parasitemia (Figure 4). However, in individual analysis one study showed a negative association, indicating that this TLR had a protective effect on high parasitemia in this population [17].

TLRs are the best-studied class of receptors in innate immune response and play a crucial role in the initial stages of various infections. In malaria, this acute phase of infection will be responsible for guiding the clinical course to mild or severe forms of the disease [6, 12]. The role of these receptors during malaria is difficult to measure due to the impact of the genetic background of the population studied [49, 50]. The genetic differences of each population make it very difficult to make any comparison between the results of studies carried out in different locations.

Considering the dynamics of the parasite-host relationship, the local genetic variability of the *Plasmodium* is also a crucial factor that will influence the recognition by the TLRs and, therefore, all the results presented here. *Plasmodium* is a complex protozoan which presents several different forms during its biological cycle, and in each one of them there is a great antigenic diversity which is exposed to the effects of the host's immune system. In this interaction with different hosts, different variants can be established in different regions of the world [51]. For *P. falciparum* in Brazil, distinct population structures were described, with *Plasmodium* differing by region or over time, even within the same country [52, 53]. The scenario for *P. vivax* is similar, with a high variability of circulating haplotypes in the world and different patterns of genetic diversity [54, 55].

Another limitation that we acknowledge is the possibility of unmeasured host and parasite genetic factors which can interplay and, therefore, co-modulate important features of malaria clinical parameters. The high heterogeneity of the included studies regarding locations, groups, the age of the individuals and the analysis criteria make it difficult to compare data.

In spite of all limitations, the present study gathered all published data seeking to examine the associations between TLRs and malaria in respect of the two main species worldwide (*P. falciparum* and *P. vivax*). In this study, we summarize all the suggested associations with important receptors of innate immune response that could impact malaria susceptibility and immunopathogenesis. Overall, the number of studies regarding this subject is small and future investigation should be undertaken in order to validate these results, including an analysis of the contribution of ethnicity in the different populations as well as the investigation of parasite variability to determine whether TLRs modulate malaria.

5. Conclusions

The meta-analysis showed that TLR1, TLR9 and TLR4 were associated with parasitemia, while TLR2 and TLR6 were related with severity and no TLR was related with susceptibility. Although promising, these results should be interpreted with caution as these studies have high genetic variability and the results could vary in different populations. Since TLRs play a crucial role in malaria's susceptibility and pathogenesis, these findings should be corroborated by performing new studies worldwide.

Declarations

Author contribution statement

Aína Danaisa Ramirez, Myrela Conceição Santos De Jesus and Júlia Rossit: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Nathália Faria Reis and Marcelo Cerilo Santos-Filho:Analyzed and interpreted the data; Wrote the paper.

Adriana Pittella Sudré, Joseli Oliveira-Ferreira, Andrea Regina de Souza Baptista and Luciane Moreno Storti-Melo: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Ricardo Luiz Dantas Machado: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Funding statement

This work was supported by Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (Brazil) and Organization of American States (EUA).

Data availability statement

No data was used for the research described in the article.

Declaration of interests statement

The authors declare no conflict of interest.

Additional information

Supplementary content related to this article has been published online at https://doi.org/10.1016/j.heliyon.2022.e09318.

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