ISSN: 0975-833X

Available online at http://www.journalcra.com

International Journal of Current Research
Vol. 11, Issue, 11, pp.7986-7992, November, 2019

DOI: https://doi.org/10.24941/ijcr.36438.11.2019

INTERNATIONAL JOURNAL OF CURRENT RESEARCH

RESEARCH ARTICLE

ROLE OF SERUM CIRCULATORY IGE AND TNF-α LEVEL AND INTERLEUKIN 4 - 590C/T POLYMORPHISM IN SUDANESE CHILDREN WITH SEVERE FALCIPARUM MALARIA

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ARTICLE INFO

Article History:
Received 14th August, 2019
Received in revised form
18th September, 2019
Accepted 25th October, 2019
Published online 26th November, 2019

Key Words:

Interleukin- 4- Single Nucleotide Polymorphism - Cerebral malaria - Severe malaria anaemia - Uncomplicated malaria - parasitaemia.

ABSTRACT

Background: Malaria, a disease with a wide variation in clinical presentation (ranging from mild to deadly cerebral malaria) remains an important health challenge in the tropics including Sudan. This study investigates the role of allelic single nucleotide polymorphism in Promoter regions (-590 C/T) of the IL-4 genes, Serum IL-4, IgE and TNF-concentrations in severity of malaria in Sudanese children. **Methods:** This is hospital based- case control cross-sectional study on one hundred ten malaria patients and sixty healthy controls. The blood samples were assessed for IL-4, IgE and TNF-α using enzyme-linked immunosorbent assay (ELISA) technique. Extracted DNA and ready use master mix were used in polymerase chain reaction, then the product was digested by BSMFI restriction enzyme. **Results:** IL-4 SNP was found to have a significant association with the development of cerebral malaria. Allele frequency was CT % = 50:50 (p = 0.03). Results shows a significant difference (p= 0.003) in IL-4 concentration when compared with allele genotypes in cerebral malaria patient, the highest concentration was shown in the allele TT (363.0 ± 199.5 Pg/ml).IL-4 level in uncomplicated malaria patients revealed a significant difference between groups (p= 0.009), the highest concentration was for mutant allele CT (428.4 ± 101.1 Pg/ml). **Conclusion:** Our study suggests that IL-4 (-590 C/T) gene polymorphism is associated with the severity of *Plasmodium falciparum* malaria infection in Sudanese children. IL-4 SNP was found to have a significant association with the development of cerebral malaria and with increased levels of serum IL-4.

Citation: Amar B Elhussein, Kawthar A Mohammedsalih, Walid G. Babikr, Omar E Fadlelseed, Soad M Alfadol5 Ashraf Naeem, NourElhouda A A Rahma and Mohammed H F Shalayel. **2019.** "Role of serum circulatory IgE and TNF-α level and Interleukin 4 - 590C/T Polymorphism in Sudanese children with Severe Falciparum Malaria", *International Journal of Current Research*, 11, (11), 7986-7992.

INTRODUCTION

Children are one of most vulnerable group affected by malaria. There were an estimated 450 000 malaria deaths around the world in 2015, of which approximately 70% were in children under 5 years of age (WHO, 2016). Cytokines are thought to play an important role in the pathogenesis of severe malaria (Prakash *et al*., 2006). Many studies have reported different cytokines as being associated either with severe malaria or with protection against severity of the disease in humans (Ho, 1998; Kurtis, 1999; Nussenblatt, 2001; Peyron *et al*., 1994).

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The balance between cytokine concentrations was found to be as important as cytokine concentration in determining the severity of malaria in infected children (Othoro *et al*., 1999). IL-4was shown to be involved in the response to *P. falciparum* malaria (Elhussein, 2018). Malaria parasitaemia, which is associated with elevated serum levels of IL-4in Sudanese children suffering from *Plasmodium falciparum* malaria, is correlated with the severity of malaria hyperparasitaemia rather than with the severity of the disease (Elhussein, 2018). Single nucleotide polymorphism of the genes control and regulatory regions may affected and governed by individual intra-variations. The production of IL-4 was shown to be increased as well as IgE concentration due to single nucleotide polymorphism in promotor region of IL-4-590 C/T

(Nakashima, 2002; Rosenwasser *et al*., 1995). The IL-4-590T, IL-4-524T and IL4-589T are corresponding alleles with an alternative numbering scheme (Gyan *et al*., 2004; Luoni *et al*., 2001; Verra *et al*., 2004). The allele IL4-524T in Fulani tribe in West Africa was shown to be associated with elevated levels of anti- *P. falciparum* IgG antibodies and with protection against malaria infection (Luoni *et al*., 2001), while IL-4-589T allele was associated with the elevated levels of total IgE in children with severe Falciparum malaria who have been living in Burkina Faso (Verra, 2004). Conversely, cerebral malaria was associated with high levels of the total IgE in children who were carried IL-4-590T in Ghana [11]. Thus the link between the malaria severity and the Single Nucleotide polymorphism of interleukin- 4 promotor regions are is yet controversial. The IgG2a and IgG3 antibodies are prevalent in polyclonal B cell activation in animal model infected with *Plasmodium chabaudi*. At that point, IL-4is moderately enhanced, while IFN-γ is markedly stimulated. In secondary IgG1-restricted response, only IL-4is produced (Tangteerawatana *et al*., 2009). According to previous findings, the IL-4-590 C/T polymorphism influences the balance between IL-4and IFN-γ and thus, could alter the severity of malaria (Duarte *et al*., 2012). When the same sets of sera were used subsequently to determine the anti- *P. falciparum* IgG subclasses and IgE antibodies, the results showed different regulations in patients with complicated and uncomplicated malaria (Tangteerawatana *et al*., 2004).

Immunoglobulin E has traditionally been associated with atopic disease and systemic anaphylaxis (Duarte *et al*., 2012). However, its role in host defense, parasitic infection and immune surveillance suggests many other potential functions (Pate, 2010). Some studies indicated that IgE may play a role in both pathogenesis and protection against malaria disease (Nacher *et al*., 2002). The mechanisms whereby IgE exerts its action is believed to be that IgE, in the form of immune complexes, will enhance nitric oxide (NO) and TNF production in monocytes through cross linking of the CD23 (the low affinity IgE receptors) on their surface, and this will lead to parasite killing (Kumsiri *et al*., 2016). People living in malaria-endemic regions develop elevated levels of IgE (Perlmann *et al*., 2000). In general, elevated levels of IgE reflect an underlying imbalance in the ratio of T helper (Th) cells in favor of Th2 cells producing interleukin-4 (IL-4) and IL-13, which are responsible for IgM/IgG switching to IgE (Calissano *et al*., 2003). Production of IL-4 by T cells from donors primed by natural malaria infection was found to be associated with elevated antibody levels specific for *P. falciparum* antigens (Perlmann *et al*., 1997)

MATERIALS AND METHODS

Study population and sampling: This observational hospital based case control cross-sectional study, took place in Sennar state which is located in South Sudan. One hundred and ten children with malaria participated in this study; with ages ranged from 6 month to 15 years. They were admitted to Sennar Teaching Hospital (Sudan). The patients were confirmed to have malaria through blood film examination. Sixty healthy children of the same age group from the same area without history of malaria infection were randomly selected as a control group.

Blood sample collection: Blood samples were collected by finger puncture technique to prepare thick and thin blood films (BF) for malaria diagnosis, and 5 ml of venous blood were collected and transferred into Ethylene Diamine Tetra Acetic acid dipotassium salts (K_2 (EDTA)) test tubes. All samples were centrifuged and the plasma was used to measure the level of interleukin-4. Drops of blood were spot in a filter paper for DNA extraction. All samples were collected before starting treatment regime. For the designation of relative parasite count, a simple code from one to four crosses is used according to the criteria mentioned by Gilles and Warrell. (+) for 1–10parasites per 100 thick film fields, (++) for 11–100 parasites per 100 thick film fields, (+++) for1–10 parasites per one thick film field and(++++) for more than 10 parasites per one thick film field (Sarangi *et al*., 2014; Ausubel *et al*., 1992).

DNA extraction: Extraction of human DNA was done using digestion proteinase K Phenol – Chloroform method from blood spot in filter paper (Ausubel, 1992).

Genotyping for cytokine allelic polymorphism: The gene that contains the polymorphism within the IL-4 promoter region was amplified by a polymerase chain reaction (PCR). The oligonucleotides 5`-ACTAGGCCTCACCTGATACG -3` and 5` -GTTGTAATGCAGTCCTCCTG -3` were used as primers under the following protocol; Initial denaturation at 95 Cº for 10 min, then 95 Cº for 50 seconds, 62 Cº for 50 second and 72 Cº for 50 second for 30 cycle. Final extension at 72 Cº for 5 min. After PCR completion, the PCR product was kept at 4 Cº for further use. The Il-4 gene was digested by the enzyme BSMFI [13], provided by (New England Biolab company). The digestion protocol was done according to manufacture manual. Allelic and genotypic frequencies distributions of the polymorphisms were evulated by Hardy-Weinberg equilibrium.

Antibody measurements: The concentration of plasma IL-4 was estimated Using enzyme- linked immunosorbent assay (ELISA) method using kits provided from Sunlong Biotech. ***Statistical analysis:*** Results of this study were statistically analyzed using (IBM SPSS Statistics program – version 21 64 bits for windows 10). Significant differences between groups were assessed by one-way ANOVA and *Chi square*. Allelic and genotypic frequencies distributions of the polymorphisms were in agreement with Hardy-Weinberg equilibrium.

RESULTS

IL-4 genotypes and allele frequencies in patients and controls: Blood film examination showed that 110 patients *(*53 males and 57 females) who were admitted to Sennar Teaching Hospital and enrolled in the study were infected with the parasite *P. falciparum*. Sixty healthy individuals (27 males and 33 females) were included in the study as a control group. The mean age of malaria cases of this study was 5.5 ± 3.8 years and 6.02 ± 4.3 years for the healthy control subjects. Out of 110 malaria patients 43 patients (39.1%) were diagnosed as having severe malaria with anaemia, 22 patients (20%) were diagnosed as cerebral malaria and 45 patients (40.9%) as uncomplicated malaria in addition to 60 healthy control persons. 30% of malaria patients had one cross (+) parasitaemia, 12.7% of patients had (++) parasitaemia, 21.8 % had (+++) parasitaemia and 35.5 % of patients had (++++) parasitaemia. IL-4 SNP was found to have a significant association with the development of cerebral malaria, Allele frequency (CT % = 50:50) and the p value was 0.03. The Genotypes CC, CT and TT showed frequencies of 3(13.6%), 16 (72%) and 3(13.6%) respectively (Table 1).

Table 1. Distribution of IL-4 genotypes in patients with severe anaemia, cerebral malaria, uncomplicated malaria, malaria group and Healthy control subjects

Study groups Severe	IL4-590 Allele genotypes				Allele frequency (C:T)%	P. Values
	CC	CT	TT	Total		
Severe Malarial Anaemia	18 (41.9%)	19 (44.2%)	6 (14.0%)	45 (100%)	63.95:36.05	0.78
Cerebral Malaria	3 (13.6%)	16 (72.7%)	3 (13.6%)	22 (100%)	50: 50	0.03*
Uncomplicated Malaria.	16 (35.6%)	21 (46.7%)	8 (17.8%)	45(100%)	63.95:36.05	0.81
Total Malaria patients	37 (33.6%)	56 (50.9%)	17 (15.5%)	110 (100%)	59.09: 40.91	0.58
Healthy Control	41 (68.3%)	17 (28.3%)	2 (3.3%)	60 (100%)	82.5:27.5	0.88
Total study population	78 (45.9%)	73 (42.9%)	19 (11.2%)	170 (100%)	67.35 : 32.65	0.76

Table 2. * Chi Square test with 2 df, P value less than 0.05 indicates inconsistency with Hardy-Weinberg Equilibrium

Study groups Severe	IL4-590Allele genotypes				Allele frequency (C:T)%	P. Values
	CC	CT	TT	Total		
(+) Parasitaemia	14 (42.4%)	13 (39.4%)	6 (18.2%)	33 (100%)	62.12:37.88	0.35
(++) Parasitaemia	3 (21.4%)	9 (64.3%)	2 (14.3%)	14 (100%)	53.57: 46.43	0.27
(+++) Parasitaemia	9 (37.5%)	13 (54.2%)	2 (8.3%)	24 (100%)	64.58:35.42	0.37
(++++) Parasitaemia	11(30.4%)	21(49.5 %)	7 (20.1%)	39 (100%)	55.13: 44.87	0.58

Table 3. Comparing IL-4, IgE and TNF- α concentrations with allele frequency in malaria cases and Healthy control subjects

Parameter	Malaria Patients			Control		
	Allele Genotypes			Allele Genotypes		
	CC	CT	TT	CC	CT	TT
IL-4 Pg/ml	106.7 ± 23.1	203.7 ± 45.4	136.7 ± 42.8	71.8 ±26.3	39.7 ±8.6	55.4 ±17.3
Sig	0.214			0.737		
IgE µg/l	315.6 ±67.0	318.8 ±51.0	245.9 ±51.2	208.8 ±13.4	209.7 ±19.8	212.5 ±101.8
Sig	0.765			0.998		
TNF-α Ng/l	334.6 ±66.4	329.2 ±54.4	341.8 ±52.4	190.4 ±24.7	218.3 ±64.2	419.4 ±122.7
Sig	0.992			0.262		

Table 4. Comparing IL-4, IgE and TNF- α concentrations with allele genotype in complications of malaria

Type of malaria	Parameter	Allele Genotypes			p. value
		CC	CT	TT	
Severe malaria anaemia	IL-4 Pg/ml	114.4 ± 37.0	70.8 ± 31.3	84.6 ± 23.0	0.633
	IgE µg/l	416.7 ± 134.3	483.7± 140.9	182.2 ± 19.3	0.516
	TNF- α Ng/l	311.3 ± 54.4	262.5 ± 33.1	392.3 ± 145.3	0.440
Celebral malaria	IL-4 Pg/ml	33.5 ± 9.1	66.8 ± 11.8	363.0 ± 199.5	0.003*
	IgE µg/l	261.9 ±37.3	236.5 ±30.3	181.0 ±59.3	0.668
	TNF- α Ng/l	154.5± 43.8	577.7± 172.8	271.9± 59.7	0.467
Uncomplicated malaria	IL-4 Pg/ml	111.9 ± 33.8	428.4 ± 101.1	90.9 ± 32.0	0.009*
	IgE µg/l	211.9±20.2	232.4±22.7	318.1±103.6	0.249
	TNF- α Ng/l	394.6± 141.2	200.1± 19.6	330.2 ± 32.9	0.235
Healthy control	IL-4 Pg/ml	71.8 ± 26.3	39.7 ± 8.6	55.4 ± 17.3	0.737
	IgE µg/l	208.8 ± 13.4	209.7 ± 19.8	212.5 ± 101.8	0.998
	TNF- α Ng/l	190.4 ± 24.7	218.3 ± 64.2	419.4 ± 122.7	0.262

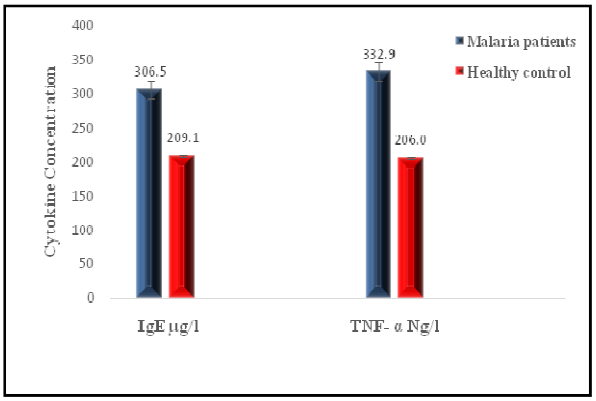

Figure 1. Serum IgE µg/l and TNF- α Ng/concentrations in malaria patients and healthy control group, where the p. value was 0.04 for IgE and 0.01 for TNF- α

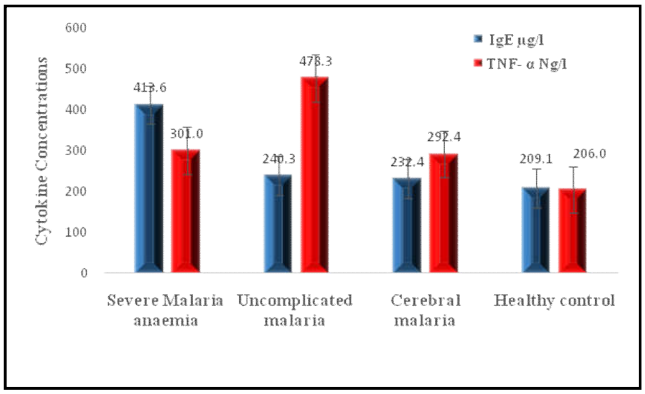

Figure 2. Serum IgE µg/l and TNF- α Ng/l concentrations in severe malaria anaemia, uncomplicated malaria, cerebral malaria patients and control group, the p. value was 0.004 for IgE and 0.011

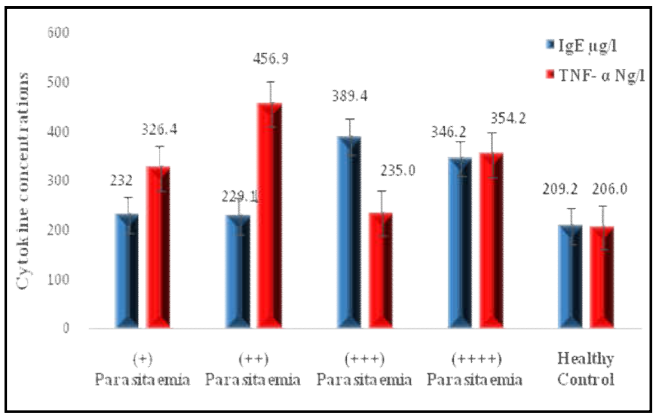

Figure 3. Serum IgE µg/l and TNF- α Ng/l concentrations in different classes of Parasitaemia and control group, the p value was 0.05 for IgE and 0.04 for TNF- α

Results displayed a non-significant association with the development of severe malarial anemia (SMA), allele frequency (CT % =63.95:36.05, p = 0.78). The frequency of genotypes CC, CT. TT were 18 (41.9%), 19 (42.2%) and 6 (14%) respectively. Polymorphism showed a non-significant association (p = 0.81) with uncomplicated malaria (UM), the allele frequency (CT % = 63.95:36.05) and the frequency of genotypes CC, CT. TT were 16 (35.6%), 21 (46.7%) and 8 (17.8%) respectively (Table 1). Moreover, a non-significant association with the (+) parasitaemia levels and IL-4 SNP genotypes was found, the allele frequency (CT % =62.12:37.88) with p value = 0.35. The frequency of genotypes CC, CT. TT were 14 (42.4%), 13 (39.4%) and 6 (18.2%) respectively. The (++) parasitaemia revealed a non-significant association with IL-4 SNP genotypes, the allele frequency (CT % =53.57: 46.43) with p value = 0.27. The frequency of genotypes CC, CT. TT were 3 (21.4%), 9 (64.3%) and 2 (14.3%) respectively**.** Polymorphism was found to have a non-significant association with the (+++) parasitaemia level, allele frequency (CT % = 64.58:35.42) and the p value was 0.37. The IL-4 SNP genotypes CC, CT and TT showed frequencies of 9(37.5%), 13(54.2%) and 2(8.3%) respectively**.** The (++++) parasitaemia level showed non-significant association with IL-4 SNP genotypes, the allele frequency (CT % =59.09: 40.91) with p. value = 0.58. The frequency of genotypes CC, CT. TT were 11 (30.4%), 21 (49.5%) and 7 (20.1%) respectively (Table 2).

Results of mean differences for concentrations of serum IL-4 with IL- 4 allele frequency for malaria cases and healthy control subjects are exhibited in Table 3. These results showed non-significant association between IL-4 SNP and serum IL-4 levels (p. value = 0.214), the highest concentration was shown in mutant allele CT with mean (203.7 ± 45.4Pg/ml) followed by heterozygote allele TT (136.7 ± 42.8Pg/ml) followed by wild allele (106.7 ± 23.1Pg/ml) and the least was in control group with mean concentration (62.1 ±140.7 Pg/ml). Results presented in Table 4 show a significant difference (p= 0.003) in IL-4 concentration when compared with allele genotypes in cerebral malaria patient. The highest concentration was shown in the allele TT (363.0 ± 199.5 Pg/ml) followed by mutant allele (66.8 ± 11.8Pg/ml) and wild allele (33.5 ± 9.1 Pg/ml). IL-4 level in uncomplicated malaria patients revealed a significant difference between groups (p= 0.009), the highest concentration was for mutant allele CT (428.4 ± 101.1Pg/ml) followed by the CC (111.9 ± 33.8Pg/ml) allele and TT allele (90.9 ± 32.0Pg/ml).

Results in Figure 1 showed there was significant difference (P < 0.04) between malaria and control groups in the concentration of IgE. The higher concentration was found in malaria group (306.5 ± 35.1 µg/l) when compared to healthy control group (209.1 ± 10.9µg/l). Results in Figure 2 showed a significant difference between the studied groups (p = 0.04) in the concentration of IgE, the higher concentration was existed in severe malaria anaemia group with mean of (413.6 ± 84.0 µg/l), followed by uncomplicated malaria (240.3 ± 22.2 µg/l), cerebral malaria group (232.4 ± 23.8 µg/l) and finally healthy control subjects with concentration (209.1 ±19.8 µg/l). Figure 3 showed significant differences in IgE concentrations among parasitaemialevel groups (p = 0.004). The highest concentration was (+++) parasitaemia (389.39± 101.7 µg/l) followed by (++++) parasitaemia group (346.2 ± 72.2 µg/l) (+) parasitaemia group (232.0 ± 27.6 µg/l) group, and (++) parasitaemia group (229.11 ± 17.29 µg/l), while the lowest concentration was seen in the control group (209.2 ± 10.9 µg/l).

DISCUSSION

The aim of this study was to determine the role of allelic single nucleotide polymorphism in the promoter regions of the interleukin-4 (-590 C/T) genes in correlation with the severity of malaria in Sudanese children, and their relation to serum lL-4 concentration in the blood of severe malaria patients. Severe anaemia and cerebral malaria complications are considered the main causes of morbidity and mortality but some evidence propose that the host's immunological response could also engage to the pathophysiology of the disease (Sarangi, 2014; Malaguarnera, 2002). Several studies of the immune response to malaria infection in human have provided a plenty of information about the cells and cytokines involved in the pathophysiology of survival and fatal outcome in severe disease (Malaguarnera, 2002). We investigated the possible linkage of disequilibrium between IL-4 polymorphisms at -590 loci within the total study material. There was a significant association between polymorphism and cerebral malaria and IL-4 SNP was found to have a significant association with the development of cerebral malaria, these results were in agreement with Gyan *et al.,* which found the mutant allele was associated with cerebral malaria (Gyan, 2004).

The study results revealed significant association between IL-4 polymorphism at -590 loci with uncomplicated malaria cases. This finding is differ from the results of Tangteerawatanaet *et al.,* were they found that the frequency of IL4-590 T allele in patients with complicated malaria did not differ from those with uncomplicated malaria (Tangteerawatana *et al*., 2004). IL-4 increases the susceptibility of individual to have cerebral malaria hence it increases the mass of the malaria parasite[26]. It also enhances infiltration of monocytes, basophils and eosinophils in addition to increasing parasite sequestration of RBCS (Cabantous *et al*., 2009), which leads to cerebral malaria and renal failure[28]. IL-4 also stimulates expression of the adhesion molecule V-CAM on endothelial cells (Schleimer *et al*., 1992), a receptor for the *P. falciparum* erythrocyte membrane protein 1(PfEMP1) involved in severe malaria pathogenesis. Up regulation of TNF has also been identified to play a role in the pathogenesis of cerebral malaria by sequestration of parasitized RBCS in endothelium of microvessels (Kwiatkowski, 1990). Thus, it is of no doubt that severe complications of malaria are due to multiple factors and

that the pathogenesis may be regulated by several mechanisms (Gyan *et al*., 2004). Current results indicated that this polymorphism increased the IL-4 level in plasma, 590T polymorphism have been shown to be associated with high level of serum IL-4 in several studies (Nakashima *et al*., 2002; Jha, 2012; Farouk, 2005). There for, the SNP-C/T transition at position -590 bp from the open reading frame has been shown to increase promoter region activity of IL-4 gene, indicating that this mutation may increase the expression of IL-4 in humans. Thence, the IL4-590 polymorphism may represent a useful prognostic marker in identifying disease severity (Rosenwasser *et al*., 1995). The concentration of IgE is significantly higher in malaria patients than in normal control group, the elevation may be instrumental in the pathogenesis of this disease (Perlmann, *et al*., 1997; Seka-Seka *et al*., 2004). Approximately 85% of children and adults living in areas of The Gambia, Liberia, Madagascar, or Thailand in which *Plasmodium falciparum* malaria is endemic have elevated levels of blood IgE, comprising both total IgE and IgE antimalarial antibodies (Perlmann, 1997). The elevation of IgE in malaria is also observed by Eze and Christian 2016, in Nigeria (Eze, 2016), and the study conducted in Thailand by Kumsiri *et al*. (Kumsiri *et al*., 2016). These results indicate that the IgE antibodies may play a role in the pathogenesis of malaria infection. Malaria infections are associated with elevations of malaria-specific and total IgE; up to 5% of total IgE is malaria specific. Antibody production and especially IgE induction reflects a switch from Th1 to Th2, due to repeated exposure of the immune system to the parasites and involves a shift from IgM/G- to IgE that increase with age till puberty (Perlmann *et al*., 1999).

Our results revealed that the levels of IgE was higher in severe malarial anaemia patients in compared to uncomplicated, cerebral malaria and control groups, this results is contrary with Verra *et al*., which found Higher levels of both total and specific *P. falciparum* IgE have been detected in cerebral malaria compared to uncomplicated malaria (Verra *et al*., 2004). This study shows non- significant difference in IgE levels between different IL-4 genotypes, this result is contrary with results obtained by Verra *et al*., which found that the IL-4 gene polymorphism was associated with significantly elevated levels of total serum IgE (Verra *et al*., 2004), also in contrast to the results recently demonstrated in Mali of West Africa. The *IL4*-590T alleles were associated with the increased levels of both total and anti-malarial IgE in the Fulani, but not the Dogon (Tangteerawatana *et al*., 2009). These results may describe the protective role of IgE in parasitic infections. In any event, IgE-mediated cellular reactions involving eosinophils, platelets or monocytes/macrophages (IgE-binding cells, equipped with Fcε receptors) have been shown to efficiently kill helminthic parasites in vitro. Such mechanisms could contribute to protection in malaria (Elghazali *et al*., 1997). On the other hand, other evidence suggests that IgE could play a role in the pathogenesis of malaria (Leoratti *et al*., 2008), the increase of the levels of IgE among individuals suffering from severe malaria in comparison to uncomplicated malaria (Seka-Seka *et al*., 2004).

Conclusion

Our study suggests that IL-4 (-590 C/T) gene polymorphism is associated with the severity of *plasmodium falciparum* malaria infection in Sudanese children. The results elucidated that this polymorphism influences IL-4 levels. IL-4 SNP has a significant causal association with the acquirement of cerebral malaria. On the other hand, the results postulate that there is an association between IL-4 SNP and degree of parasitaemia. IgE play a significant role in pathogenesis of malaria. Severe malaria anaemia is associated with high level of IgE. Our results suggest the role of IgE in severity of malaria disease. The IgE is associated with hyperparasitaemia.

Grant information: No Fund.

Conflict of interest: No conflicts of interest.

Ethics approval: All patients were assured that all their obtained information was handled in a confidentialatmosphere and will not adversely affect their lives after taking verbal and written consents

Acknowledgments

Our sincere thanks and appreciation go to the children who participated in this study and their families who accepted the request for participation. We also thank the family of Virology lab, Central laboratory (Ministry of Higher Education) who welcomed us and gave us the opportunity to join their team, and gave us an access to the laboratory and research facilities.

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