ORIGINAL RESEARCH

Associations of Single Nucleotide Polymorphisms in IL-18 Gene with *Plasmodium falciparum*-Associated Malaria

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Introduction: Interleukin-18 (IL-18) is a pro-inflammatory cytokine, reported to be involved in the initial immune responses against malaria. Genetic variations in the host are an important factor that influences the etiology of malaria at several disease levels. Polymorphisms within the *IL-18* gene are associated with susceptibility and clinical outcome of several diseases.

Methods: We genotyped single nucleotide polymorphisms (SNPs) in *IL-18* of patients infected with *Plasmodium falciparum* with varying extent of parasitemia and different age groups.

Results: SNPs rs5744292 (OR = 70.446; 95% CI = 4.318–1149.323; p<0.0001) and rs544354 (OR = 1.498; 95% CI = 1.088–2.063; p=0.013) were found to be significantly associated with parasitemia in *P. falciparum*-infected patients when compared with healthy control subjects. SNP rs5744292 (OR = 7.597; 95% CI=1.028–56.156; p=0.019) was associated with increased parasite density in infected patients. SNPs rs544354 (OR 0.407; 95% CI=0.204–0.812; p = 0.009) and rs360714 (OR of 0.256; 95% CI=0.119–0.554; p = 0.001) were significantly associated with parasite density in an age-dependent manner, with the risk alleles present more frequently among the younger (1–9 years) patients. Several haplotypes were found to have a significant association with parasitemia. In-vitro expression analysis using luciferase reporter assay showed that SNPs rs1946518 and rs187238 in the *IL-18* gene promoter region and rs360728 and rs5744292 in the 3′-untranslated region of the *IL-18* gene were associated with enhanced transcriptional activity.

Conclusion: Our results suggest that polymorphisms within the *IL-18* gene are associated with the susceptibility to *P. falciparum* infection and related parasitemia among groups with different parasite density and across various age groups.

Keywords: *Plasmodium falciparum*, *IL-18* gene, SNPs

Introduction

Malaria is a parasitic disease caused by the unicellular protozoan of the genus *Plasmodium* and is spread mainly through infected female *Anopheles* mosquitoes. Despite several control programs, malaria continues to be a major health concern worldwide, particularly in low and middle-income countries. The WHO estimates there were 228 million cases of malaria globally resulting in 405,000 deaths in 2018.[1] Malaria is presently widespread along the tropical and sub-tropical belt, in certain areas of the Americas, many parts of Asia, and much of Africa, particularly sub-Saharan Africa.[2] Malaria has been known to be endemic in the lowlands of

Saudi Arabia, and three species of *Anopheles* mosquitoes have been identied in this region, namely *An. arabiensis*, *An. Sergentii* and *An. Gambiae*.[3,4] Among the different species of *Plasmodium* infecting humans, *Plasmodium falciparum* is the most prevalent in African countries and Saudi Arabia and it is linked to most of the malaria-related deaths worldwide.[5,6]

Large variation has been observed in the clinical presentation of the *P. falciparum* infection, from asymptomatic individuals to patients with severe cerebral malaria and other fatal forms of the infection. The individual variations in the clinical manifestation and progression to severe forms of *P. falciparum* infection have been reported to be influenced by several factors such as age, genetic polymorphisms in the host, genotype of the parasite, and the environmental/social conditions.[7,8] Numerous studies have explored genetic influence in the pathomechanism and identified several host genes that can significantly influence the diverse clinical outcomes of malaria such as hemoglobin variants,[9,10] glucose-6-phosphate dehydrogenase,[11] pyruvate kinase,[12] and haptoglobin.[13]

Cytokines orchestrate the initial immune response against invading malarial parasites. Previous studies have reported an increased concentration of pro-inflammatory cytokines like IL-6 and anti-inflammatory cytokines like IL-10 in *P. falciparum*-infected patients.[14] IL-18 is a pro-inflammatory cytokine encoded by the *IL-18* gene on chromosome 11q23.1 and belongs to the IL-1 superfamily. IL-18 is expressed by a wide range of cells, including macrophages, Kupffer cells, keratinocytes, osteoblasts, astrocytes, and dendritic cells (DCs).[15] IL-18 is synthesized as 24 kD pro-IL-18, a biologically inactive precursor peptide, which is subsequently cleaved by caspase-1 into a biologically active IL-18 cytokine.[15] The most salient biological property of IL-18 that distinguishes it from IL-1 is its ability to induce secretion of high levels of interferon (IFN)–γ in the presence of IL-12.[16] Also, a substantial pool of intracellular IL-18 is present in the cytoplasm and is regulated mainly by caspase-1-mediated pro-IL-18 processing.[15] IL-18 has a wide array of biological functions, many of which are accomplished synergistically with other cytokines, such as IFN-γ, as an early immune response to pathogenic invasions.[16] Several investigations into biological role of IL-18 have shown that IL-18 is a potent inducer of (i) tumor necrosis factor alpha (TNFα), Fas-ligand, and IL-2 from T and NK cells; (ii) IL-1b, IL-8, and macrophage inflammatory protein (MIP)-1α from

peripheral blood mononuclear cells; and iii) cyclooxygenase (COX2), and inducible nitric oxide synthase.[17–19]

The initial immune response to *P. falciparum* infection is mediated by IFN-γ that is induced by the combination of interleukin 12 (IL-12) and 18 (IL-18). This cytokine secretion is crucial for the control of parasitemia and resolution of malarial infection through TNFα induction and enhanced release of reactive nitrogen and oxygen radicals.[20] Torre et al (2001) demonstrated that IL-18 may have a pro-inflammatory role in patients with uncomplicated *P. falciparum* infection.[21] In another study, it was found that IL-18 and IL-12 levels were higher in children with mild malaria than in children with a severe form of the disease.[22] Also, high quantities of both IL-18 and IFN-γ molecules was found to be associated with severe forms of malarial infections.[20] A study in western Kenya investigated the relationship between IL-18 and clinical malaria phenotypes in children, and reported association between higher secretion of IL-18 with severe malarial anemia (SMA).[23] Similarly, a study in adults demonstrated a close association between increased IL-18 levels and severe *falciparum* malaria.[24] Taken together, these findings indicate that IL-18 is an important factor in the pathogenesis of *P. falciparum* induced malaria.

Several polymorphisms within the *IL-18* gene have been reported to play an important role in the etiology of several diseases such as atherosclerosis,[25] rheumatoid arthritis (RA), systemic lupus erythematosus (SLE),[26,27] allergic asthma, and allergic dermatitis,[28] mostly by altering the circulating levels of IL-18. However, very few studies have investigated *IL-18* gene polymorphisms in relation to its role in malaria pathogenesis.

Previously, we have shown that genetic variations in the *IL-22* gene could be correlated with susceptibility to malaria infection.[29] In this study, we investigated whether the single nucleotide polymorphisms (SNPs) spanning the *IL-18* gene were associated with the susceptibility to *P. falciparum* infection and related parasitemia. We also analyzed the influence of patient age and genetic polymorphisms in relation to extent of *P. falciparum* parasitemia.

Materials and Methods
Ethical Considerations
Ethical approval to conduct this study was granted by Ethical Review Board (ERB) of King Fahad Central Hospital (KFCH), Jazan (Registry no. 041). The study followed ethical guidelines set by the Helsinki

Declaration of 1975. Prior to study subject enrolment, informed consent was obtained from each subject; legal guardians provided consent on behalf of children. All patient-related research data including human biological samples were de-identified and secured to preserve the privacy of study participants.

Study Design and Population

This case–control study was conducted and a total of 450 subjects were enrolled. Blood samples were collected from 250 patients diagnosed with *P. falciparum* malaria infection admitted at Malaria Center, Jazan region, situated in the Southwest part of the Saudi Arabia. In addition, 200 healthy controls were recruited as a control cohort. We used WHO manual ("plus system" scale) to identify the parasite density in a thick blood smear from infected individuals.[30] The *P. falciparum* infected malaria subjects were classified into four categories based on the parasite density; 1) *Group I*: presence of 1–10 malarial parasites per 100 thick blood smear fields; 2) *Group II*: presence of 11–100 malarial parasites per 100 thick blood smear fields; 3) *Group III*: presence of 1–10 malarial parasites per single thick blood smear field; and 4) *Group IV*: presence of over 10 malarial parasites per single thick blood smear field. Patients were excluded from the study if they were co-infected with Dengue virus (DENV), Hepatitis C virus (HCV), Hepatitis B virus (HBV) or Human immunodeficiency virus (HIV).

Selection and Genotyping of IL-18 SNPs

The *IL-18* gene is made up of 20.9 kb and consists of a 5′-untranslated region (UTR) promoter, 6 exons and a 3′UTR region. The SNPs were genotyped using the polymerase chain reaction (PCR)-based direct DNA sequencing and SNP-specific TaqMan assays. Ten tag SNPs were identified based on the following criteria: minor allele frequency (MAF) more than 5% and linkage disequilibrium (LD) threshold of $r^2 \geq 0.8$. The 10 identified tag SNPs were: rs11214101, rs544354, rs360729, rs5744260, rs549908, rs360714, rs7106524, rs5744231, rs360718, and rs1293344. In addition, six SNPs, rs187238, rs1946518 and rs1946519 from the promoter region and rs360727, rs5744292 and rs360728 from and 3′UTR of the *IL-18* gene were selected. The details of the SNPs are summarized in Figure 1 and Supplementary Table 1.

DNA Extraction and Polymerase Chain Reaction (PCR)

Using the manufacturer's guide, DNA was extracted via the DNeasy® Blood & Tissue Kit (Qiagen, Hilden, Germany). The PCR was carried out using GoTaq® Master Mixes (Promega Corporation, Madison, WI, USA). Primers were designed through the Primer3 Input version 0.4.0 available at (http://bioinfo.ut.ee/primer3-0.4.0/) and they are shown in Supplementary Table 2. All primers were M13-tailed to improve sequencing of the PCR products. The resultant PCR products were run on
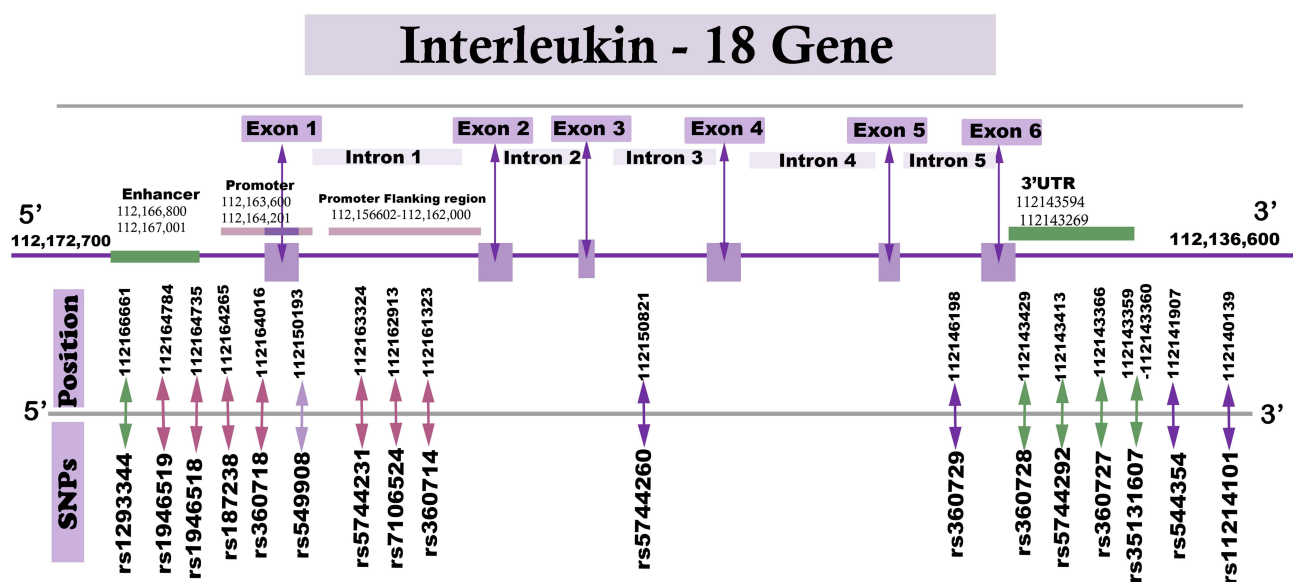

Figure 1 Schematic drawing illustrating the positions of IL-18 SNPs investigated in this study.

2% agarose gel electrophoresis to ensure PCR products' quality. The amplified PCR products underwent direct sequencing by a BigDye™ Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) as per the recommended protocol. Afterward, each of the PCR products was subjected to both reverse and forward sequencing. The final sequences were evaluated and modified using the SeqMan Pro 15 Lasergene (DNASTAR, Inc., Madison, WI, USA).

Transfection and Luciferase Reporter Assay

Several DNA fragments of the *IL-18* promoter and 3′ UTR region were cloned and transfected in HuH7 (obtained from the Japanese Collection of Research Bioresources Cell Bank, catalogue number: JCRB0403) cells and the variations in the luciferase gene activity were monitored. The chromosomal positions of the fragments and the primers used for amplification are shown in Supplementary Tables 3 and 4 for the promoter and 3′ UTR regions, respectively. All of the fragments were cloned in pGL3 Basic Vector (accession number: U47297) (Promega, Madison, WI, USA). HotStar DNA Polymerase (Qiagen, Hilden, Germany) was used for PCR amplification using the guidelines of the manufacturer. For colony selection on ampicillin plates, Escherichia coli DH5α competent cells (catalogue number: 18265017) (ThermoFisher Scientific, Sunnyvale, CA, USA) were utilized. Replicates were selected and DNA was extracted through QIAamp DNA Mini Kit (Qiagen, Valencia, CA). Extracted DNA fragments were then transfected into HuH7 cells, which were cultivated and evaluated for luciferase activity after 48 hours.

Statistical Analyses

We used HaploView software version 4.0 (Broad Institute of MIT and Harvard, Cambridge, MA, USA) to analyze SNPs and measure linkage disequilibrium (LD), haplotype frequencies and MAFs. To assess the genotypic association for selected genetic variants, we used the De Finetti program available at (http://ihg.gsf.de/cgi-bin/hw/hwa1.pl). In addition, we conducted analyses to identify for the allelic, recessive and dominant modes of inheritance for each genetic variant. The study findings were presented as odds ratios (OR) with their 95% confidence intervals (CI). The Hardy–Weinberg equilibrium (HWE) was performed to test for deviation; *P* value <0.05 was considered as a statistically significant deviation from HWE. The *P* value <0.05, obtained in a two-tailed test, was set as a threshold for statistically significant association.

Results

Genotype and Allele Frequency Analysis Between *P. falciparum*-Infected Patients and Healthy Control Group

Genotypic distributions of SNPs in the healthy control group and the *P. falciparum*-infected patients are shown in Table 1. We found that SNPs rs5744292 (OR = 70.446; 95% CI= 4.318–1149.323; p<0.0001) and rs544354 (OR = 1.498; 95% CI= 1.088–2.063; p = 0.013) were of significantly higher occurrence in *P. falciparum*-infected patients when compared to healthy control group. The rs5744292-G allele (8%) and rs544354-A allele (26%) showed increased frequency among infected patients in comparison with the control group. We also observed that the individuals carrying either the homozygous genotype rs5744292-GG or the heterozygous AG were associated with increased risk of malarial infection with an OR of 13.828 and 48.857, respectively, when compared to homozygous AA carriers. Under a dominant model of inheritance, the rs5744292-G allele was found to be associated with an increased risk of infection (OR = 61.763). Similarly, we found that individuals carrying the homozygous AA genotype with SNP rs544354 had an increased risk of parasitemia (OR = 5.253) when compared to homozygous GG carrying individuals. In addition, the rs544354-A allele was significantly associated with risk of *P. falciparum* infection in both dominant (OR = 1.435) and recessive (OR = 0.209) models. The homozygous SNP rs360714 GG genotype was found to be associated with an increased risk of malaria infection (OR = 0.147) compared to the homozygous AA genotype, whereas the heterozygous rs7106524 AG genotype was associated with an increased risk of the infection (OR = 1.652) compared to the homozygous GG genotype.

Genotype and Allele Frequency Analysis Based on Parasite Density

Patients were divided into 4 groups based on increasing parasite density: Group 1 (lowest density; 14 patients), Group 2 (24 patients), Group 3 (52 patients), and Group 4 (highest density; 160 patients). The 16 selected polymorphisms were further investigated to determine their association with varying levels of parasitemia in the *P.*

Table 1 Genotype Distribution and Allele Frequency of *IL-18* Gene SNPs Among *P. falciparum*-Infected Patients and Healthy Control Subjects

SNPs	Genotype/ Allele	Controls n=200	Patients n=250	OR (95% C.I.)	χ^2	P-value*
rs1946518	GG	55 (27.5%)	78 (31.2%)	Ref		
	GT	100 (50%)	120 (48%)	0.846 (0.547–1.308)	0.57	0.452
	TT	45 (22.5%)	52 (20.8%)	0.815 (0.481–1.381)	0.58	0.446
	G	210 (52.5%)	276(55.2%)	0.897 (0.689–1.168)	0.65	0.419
	T	190 (47.5%)	224(44.8%)			
	TT+GT.vs.GG			0.836 (0.56–1.26)	0.73	0.393
	TT.vs.GT+GG			1.105 (0.70–1.73)	0.19	0.663
rs1946519	CC	55 (27.5%)	82 (32.8%)	Ref		
	AC	103 (51.5%)	119 (47.6%)	0.775 (0.503–1.193)	1.34	0.246
	AA	42 (21%)	49 (19.6%)	0.783 (0.458–1.337)	0.81	0.368
	C	213 (53.25%)	283 (56.6%)	0.873 (0.671–1.138)	1.01	0.315
	A	187 (46.675%)	217 (43.4%)			
	AA+AC.vs.CC			0.777 (0.517–1.168)	1.47	0.225
	AA.vs.AC+CC			1.090(0.687–1.730)	0.13	0.713
rs187238	CC	122 (61%)	148 (59.2%)	Ref		
	CG	64 (32%)	81 (32.4%)	1.043 (0.695–1.566)	0.04	0.838
	GG	14 (7%)	21 (8.4%)	1.236 (0.603–2.534)	0.34	0.561
	C	308 (77%)	377 (75.4%)	1.092 (0.802–1.488)	0.31	0.576
	G	92 (23%)	123 (24.6%)			
	GG+CG.vs.CC			1.078 (0.737–1.576)	0.15	0.698
	GG.vs.CG.CC			0.821 (0.406–1.658)	0.3	0.582
rs360728	CC	151 (75.5%)	197 (78.8%)	Ref		
	CG	37 (18.5%)	44 (17.6%)	0.912 (0.561–1.482)	0.14	0.708
	GG	12 (6%)	9 (3.6%)	0.575 (0.236–1.400)	1.52	0.218
	C	339 (84.75%)	438 (87.6%)	0.787 (0.538–1.151)	1.53	0.216
	G	61 (15.25%)	62 (12.4%)			
	GG+CG.vs.CC			0.829 (0.533–1.291)	0.69	0.406
	GG.vs.CG+GG			1.709 (0.705–4.142)	1.44	0.230

(Continued)

Table 1 (Continued).

SNPs	Genotype/ Allele	Controls n=200	Patients n=250	OR (95% C.I.)	χ^2	P-value*
rs5744292	AA	200 (100%)	217 (86.8%)	Ref		
	AG	0 (0%)	26 (10.4%)	48.857 (2.958–806.993)	22.73	**<0.0001**
	GG	0 (0%)	7 (2.8%)	13.828 (0.785–243.666)	6.35	**0.012**
	A	400 (100%)	460 (92%)	70.446 (4.318–1149.323)	33.49	**<0.0001**
	G	0 (0%)	40 (8%)			
	GG+AG.vs.AA			61.763 (3.760–1014.661)	28.49	**<0.0001**
	GG.vs.AG+AA			0.081 (0.005–1.426)	5.69	**0.017**
rs360727	GG	149 (74.5%)	196 (78.4%)	Ref		
	AG	41 (20.5%)	45 (18%)	0.834 (0.520–1.340)	0.56	0.453
	AA	10 (5%)	9 (3.6%)	0.684 (0.271–1.726)	0.65	0.419
	G	339 (84.75%)	437 (87.4%)	0.801 (0.548–1.171)	1.31	0.252
	A	61 (15.25%)	63 (12.6%)			
	AA+AG.vs.GG			0.805 (0.519–1.247)	0.94	0.331
	AA.vs.AG+AA			1.409 (0.561–3.538)	0.54	0.463
rs549908	TT	120 (60%)	152 (60.8%)	Ref		
	GT	69 (34.5%)	78 (31.2%)	0.892 (0.597–1.335)	0.31	0.579
	GG	11 (5.5%)	20 (8%)	1.435 (0.662–3.112)	0.85	0.357
	T	309 (77.25%)	382 (76.4%)	1.049 (0.768–1.433)	0.09	0.764
	G	91 (22.75%)	118 (23.6%)			
	GG+GT.vs.TT			0.967 (0.661–1.414)	0.03	0.863
	GG.vs.GT+TT			0.669 (0.313–1.432)	1.08	0.298
rs11214101	TT	200 (100%)	249 (99.6%)	Ref		
	CT	0 (0%)	1 (0.4%)	2.411 (0.098–59.500)	0.80	0.370
	CC	0 (0%)	0 (0%)	0.804 (0.016–40.678)	Nan	1.000
	T	400 (100%)	499 (99.8%)	2.405 (0.098–59.206)	0.80	1.234
	C	0 (0%)	1 (0.2%)			
	CC+CT.vs.TT			2.411 (0.098–59.500)	0.80	0.370
	CC.vs.CT+TT			1.249 (0.025–63.242)	Nan	1.000

rs544354					
GG	127 (63.5%)	137 (54.8%)	Ref	1.45	0.229
AG	70 (35%)	96 (38.4%)	1.271 (0.860–1.880)	8.21	**0.004**
AA	3 (1.5%)	17 (6.8%)	5.253 (1.504–18.351)	6.17	0.013
G	324 (81%)	370 (74%)	1.498 (1.088–2.063)		
A	76 (19%)	130 (26%)			
AA+AG.vs.GG			1.435 (0.981–2.100)	3.47	**0.006**
AA.vs.AG+AA			0.209 (0.060–0.723)	7.35	**0.007**
rs360729					
AA	126 (63%)	152 (60.8%)	Ref	0.09	0.768
AT	60 (30%)	77 (30.8%)	1.064 (0.705–1.606)	0.36	0.550
TT	14 (7%)	21 (8.4%)	1.243 (0.607–2.545)	0.41	0.524
A	312 (78%)	381 (76.2%)	1.107 (0.809–1.515)		
T	88 (22%)	119 (238%)			
TT+AT.vs.AA			1.098 (0.748–1.610)	0.23	0.633
TT.vs.AT+AA			0.821 (0.406–1.658)	0.30	0.582
rs5744260					
GG	193 (96.5%)	239 (95.6%)	Ref	0.03	0.856
AG	7 (3.5%)	11 (4.4%)	0.914 (0.346–2.412)	Nan	1.000
AA	0 (0%)	0 (%)	0.582 (0.011–29.518)	0.03	0.857
G	393 (98.25%)	489 (97.8%)	0.916 (0.351–2.389)		
A	7 (1.75%)	11 (2.2%)			
AA+AG.vs.GG			0.914 (0.346–2.412)	0.03	0.856
AA.vs.AG+AA			1.710 (0.034–86.631)	Nan	1.000
rs360714					
AA	142 (71%)	193 (77.5%)	Ref	1.30	0.254
AG	53 (26.5%)	56 (22.4%)	0.777 (0.504–1.199)	4.03	**0.045**
GG	5 (2.5%)	1 (0.4%)	0.147 (0.017–1.273)	3.29	0.069
A	337 (84.25%)	442 (88.4%)	0.702 (0.478–1.030)		
G	63 (15.75%)	58 (11.6%)			
GG+AG.vs.AA			0.723 (0.473–1.106)	2.25	0.134
GG.vs.AG+AA			6.385 (0.740–55.097)	3.72	0.054
rs7106524					
GG	92 (46%)	97 (38.8%)	Ref	5.44	**0.019**
AG	62 (31%)	108 (43.2%)	1.652 (1.082–2.522)	0.09	0.769
AA	46 (23%)	45 (18%)	0.928 (0.563–1.530)	0.11	0.737
G	246 (61.5%)	302 (60.4%)	1.047 (0.800–1.371)		
A	154 (38.5%)	198 (39.6%)			
AA+AG.vs.GG			1.344 (0.922–1.959)	2.36	0.124
AA.vs.AG+AA			1.361 (0.858–2.158)	1.72	0.189

(Continued)

Table 1 (Continued).

SNPs	Genotype/ Allele	Controls n=200	Patients n=250	OR (95% C.I.)	χ^2	P-value*
rs5744231	CC	194 (97%)	241 (96.4%)	Ref		
	CT	5 (2.5%)	8 (3.2%)	1.288 (0.415–4.000)	0.19	0.661
	TT	1 (0.5%)	1 (0.4%)	0.805 (0.050–12.953)	0.02	0.878
	C	393 (98.25%)	490 (98%)	1.146 (0.432–3.038)	0.07	0.784
	T	7 (1.75%)	10 (2%)			
	TT+CT.vs.CC			1.207 (0.422–3.451)	0.12	0.724
	TT.vs.CT+CC			1.251 (0.078–20.130)	0.03	0.874
rs360718	AA	121 (60.5%)	146 (58.4%)	Ref		
	AC	65 (32.5%)	80 (32%)	1.020 (0.679–1.531)	0.01	0.924
	CC	14 (7%)	24 (9.6%)	1.421 (0.704–2.866)	0.97	0.325
	A	307 (76.75%)	372 (74.4%)	1.136 (0.836–1.544)	0.66	0.416
	C	93 (23.25%)	128 (25.6%)			
	CC+AC.vs.AA			1.091 (0.747–1.594)	0.2	0.652
	CC.vs.AC+AA			0.709 (0.357–1.409)	0.97	0.324
rs1293344	AA	121 (60.5%)	145 (58%)	Ref		
	AG	64 (32%)	85 (34%)	1.108 (0.740–1.661)	0.25	0.618
	GG	15 (7.5%)	20 (8%)	1.113 (0.546–2.267)	0.09	0.769
	A	306 (76.5%)	375 (75%)	1.085 (0.798–1.475)	0.27	0.602
	G	94 (32.5%)	125 (25%)			
	GG+AG.vs.AA			1.109 (0.759–1.620)	0.29	0.592
	GG.vs.AG+AA			0.932 (0.464–1.872)	0.04	0.844

Note: *Bold indicates significance.

Table 2 Genotype Distribution and Allele Frequency of *IL-18* Gene SNPs When Group 1+2 of Parasite Density Was Compared with Group 3+ 4

SNPs	Genotype/Allele Distribution	Group (1+2) n=38	Group (3+4) n=212	OR (95% C.I.)	χ^2	P-value*
rs360727	GG	27 (71%)	169 (79.7%)	Ref		
	AG	10 (26.3%)	35(16.5%)	0.559 (0.248–1.259)	2.01	0.156
	AA	1 (2.7%)	8(3.8%)	1.278 (0.154–10.629)	0.05	0.819
	G	64 (84.2%)	373(87.98%)	0.729 (0.369–1.443)	0.83	0.362
	A	12 (15.8%)	51 (12.02%)			
	AA+AG.vs.GG			0.625(0.287–1.358)	1.43	0.232
	AA.vs.AG+AA			0.689 (0.084–5.674)	0.12	0.727
rs549908	TT	22(57.9%)	130(61.32%)	Ref		
	GT	15(39.5%)	63(29.71%)	0.711 (0.345–1.463)	0.86	0.325
	GG	1(2.6%)	19(8.97%)	3.215 (0.409–25.254)	1.37	0.241
	T	59(77.6%)	323(76.18%)	1.085 (0.605–1.946)	0.08	0.783
	G	17(22.4%)	101(23.82%)			
	GG+GT.vs.TT			0.867 (0.430–1.748)	0.16	0.690
	GG.vs.GT+TT			0.275 (0.036–2.114)	1.75	0.185
rs11214101	TT	38(100%)	211(99.5%)	Ref		
	CT	0(0%)	1(0.5%)	0.546 (0.022–13.654)	0.18	0.671
	CC	0(0%)	0(0%)	0.182 (0.004–9.313)	Nan	1
	T	76(100%)	423(99.76%)	0.542 (0.022–13.426)	0.18	1.014
	C	0(0%)	1(0.24%)			
	CC+CT.vs.TT			0.546 (0.022–13.654)	0.18	0.671
	CC.vs.CT+TT			5.519 (0.108–282.36)	Nan	1
rs544354	GG	20(52.6%)	117(55.2%)	Ref		
	AG	15(39.5%)	81(38.2%)	0.923 (0.446–1.910)	0.05	0.829
	AA	3(7.9%)	14(6.6%)	0.798 (0.210–3.029)	0.11	0.739
	G	55(72.4%)	315(74.3%)	0.906 (0.524–1.568)	0.12	0.724
	A	21(27.6%)	109(25.7%)			
	AA+AG.vs.GG			0.902 (0.452–1.802)	0.09	0.770
	AA.vs.AG+AA			1.212 (0.331–4.438)	0.08	0.770

(*Continued*)

Table 2 (Continued).

SNPs	Genotype/Allele Distribution	Group (1+2) n=38	Group (3+4) n=212	OR (95% C.I.)	χ^2	P-value*
rs360729	AA	21(55.3%)	131(61.8%)	Ref		
	AT	16(42%)	61(28.8%)	0.611 (0.298–1.253)	1.83	0.176
	TT	1(2.7%)	20(9.4%)	3.206 (0.408–25.16)	1.36	0.243
	A	58(76.3%)	323(76.2%)	1.008 (0.567–1.789)	0	0.979
	T	18(23.7%)	101(23.8%)			
	TT+AT.vs.AA			0.764 (0.380–1.533)	0.58	0.447
	TT.vs.AT+AA			0.259 (0.034–1.993)	1.94	0.163
rs5744260	GG	35 (92.1%)	204 (96.2%)	Ref		
	AG	3 (7.9%)	8 (3.8%)	0.458 (0.116–1.809)	1.3	0.254
	AA	0 (0%)	0 (0%)	0.174 (0.003–8.891)	Nan	1
	G	73 (96.05%)	416 (98.1%)	0.468 (0.121–1.805)	1.27	0.225
	A	3 (3.95%)	8 (1.9%)			
	AA+AG.vs.GG			0.458 (0.116–1.809)	1.3	0.254
	AA.vs.AG+AA			5.519 (0.108–282.3)	Nan	1
rs1946518	GG	9 (23.7%)	69 (32.5%)	Ref		
	GT	20 (52.6%)	100 (47.2%)	0.652 (0.280–1.517)	0.99	0.318
	TT	9 (23.7%)	43 (20.3%)	0.623 (0.229–1.693)	0.87	0.350
	G	38 (50%)	238 (56.1%)	0.782 (0.479–1.274)	0.98	0.322
	T	38 (50%)	186 (43.9%)			
	TT+GT.vs.GG			0.643 (0.289–1.4333)	1.18	0.277
	TT.vs.GT+GG					
rs1946519	CC	12 (31.6%)	70 (33.02%)	Ref		
	AC	16 (42.1%)	103 (48.58%)	1.104 (0.492–2.475)	0.06	0.810
	AA	10 (26.3%)	39 (18.4%)	0.669 (0.265–1.688)	0.73	0.392
	C	40 (52.6%)	243 (57.3%)	0.828 (0.507–1.350)	0.57	0.448
	A	36 (47.4%)	181 (42.7%)			
	AA+AC.vs.CC			0.936 (0.446–1.965)	0.03	0.861
	AA.vs.AC+CC			1.584 (0.711–3.530)	1.28	0.257

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			OR (95% CI)	χ²	P
rs187238					
CC	18 (47.36%)	130(61.32%)	Ref		
CG	19 (50%)	62(29.24%)	0.452 (0.222–0.921)	4.93	**0.026**
GG	1 (2.63%)	20(9.43%)	2.769 (0.350–21.901)	1.01	0.315
C	55 (72.36%)	322(76%)	0.830 (0.479–1.438)	0.44	0.505
G	21 (27.63%)	102(24%)			
GG+CG.vs.CC			0.568 (0.284–1.137)	2.6	0.107
GG.vs.CG.CC			0.259 (0.034–1.993)	1.9	0.163
rs360728					
CC	29 (76.3%)	168 (79.25%)	Ref		
CG	9 (23.7%)	35 (16.5%)	0.671 (0.292–1.542)	0.89	0.345
GG	0 (0%)	9 (4.25%)	3.326 (0.188–58.703)	1.54	0.214
C	67 (88.2%)	371 (87.5%)	1.063 (0.501–2.258)	0.03	0.872
G	9 (11.8%)	53 (12.5%)			
GG+CG.vs.CC			0.844 (0.372–1.913)	0.17	0.684
GG.vs.CG+GG			0.278 (0.016–4.880)	1.67	0.195
rs5744292					
AA	37 (97.36%)	180 (84.90%)	Ref		
AG	1(2.64%)	25(11.79%)	5.139 (0.675–39.122)	3.07	0.079
GG	0 (%)	7(3.30%)	3.116 (0.174–55.751)	1.43	0.231
A	75(98.69%)	385(90.80%)	7.597 (1.028–56.156)	5.44	**0.019**
G	1(1.31%)	39(9.20%)			
GG+AG.vs.AA			6.578 (0.871–49.662)	4.37	**0.036**
GG.vs.AG+AA			0.356 (0.020–6.360)	1.29	0.255
rs360714					
AA	31 (81.5%)	162 (76.41%)	Ref		
AG	6 (15.79%)	50 (23.59%)	1.595 (0.629–4.041)	0.98	0.321
GG	1(2.63%)	0 (0%)	0.065 (0.003–1.623)	5.09	**0.024**
A	68 (89.47%)	374 (88.20%)	1.136 (0.516–2.503)	0.1	0.750
G	8 (10.53%)	50 (11.80%)			
GG+AG.vs.AA			1.367 (0.567–3.293)	0.49	0.484
GG.vs.AG+AA			17.00 (0.68–425.21)	5.6	**0.017**
rs7106524					
GG	16 (42.1%)	81 (38.2%)	Ref		
AG	15 (39.5%)	93 (43.87%)	1.225 (0.570–2.631)	0.27	0.603
AA	7 (18.4%)	38 (17.92%)	1.072 (0.407–2.824)	0.02	0.887
G	47 (61.8%)	255 (60.14%)	1.074 (0.650–1.774)	0.08	0.780
A	29 (38.2%)	169 (39.86%)			
AA+AG.vs.GG			1.176 (0.583–2.371)	0.21	0.649
AA.vs.AG+AA			1.034 (0.424–2.523)	0.01	0.941

(Continued)

Table 2 (Continued).

SNPs	Genotype/Allele Distribution	Group (1+2) n=38	Group (3+4) n=212	OR (95% C.I.)	χ^2	P-value*
rs5744231	CC	38 (100%)	203 (95.8%)	Ref		
	CT	0 (0%)	8 (3.7%)	3.216 (0.182–56.889)	1.49	0.222
	TT	0 (0%)	1 (0.5%)	0.568 (0.023–14.192)	0.19	0.665
	C	76 (100%)	414 (97.6%)	3.876 (0.225–66.836)	1.83	0.372
	T	0 (0%)	10 (2.4%)			
	TT+CT.vs.CC			3.595 (0.205–63.057)	1.67	0.195
	TT.vs.CT+CC			1.176 (0.583–2.371)	0.21	0.649
rs360718	AA	20 (52.63%)	126 (59.434%)	Ref		
	AC	14 (36.84%)	66 (31.132%)	0.748 (0.355–1.576)	0.58	0.444
	CC	4 (10.53%)	20 (9.434%)	0.794 (0.246–2.564)	0.15	0.698
	A	54 (71.1%)	318 (75%)	0.818 (0.476–1.407)	0.53	0.467
	C	22 (28.9%)	106 (25%)			
	CC+AC.vs.AA			0.758 (0.379–1.517)	0.61	0.433
	CC.vs.AC+AA			1.129 (0.363–3.509)	0.04	0.833
rs1293344	AA	18(47.37%)	127(59.91%)	Ref		
	AG	19(50%)	66(31.13%)	0.492 (0.242–1.001)	3.92	**0.047**
	GG	1(2.63%)	19(89.96%)	2.693 (0.340–21.353)	0.95	0.330
	A	55(72.37%)	320(75.47%)	0.851 (0.491–1.474)	0.33	0.565
	G	21(27.63%)	104(24.53%)			
	GG+AG.vs.AA			0.602 (0.301–1.205)	2.08	0.149
	GG.vs.AG+AA			0.275 (0.036–2.114)	1.75	0.185

Note: *Bold indicates significance.

falciparum-infected individuals. Allelic and genotypic frequency comparisons were done between the patients in group 1+2 with the patients in group 3+4 (Table 2). We found that SNP rs5744292 (OR = 7.597; 95% CI = 1.028–56.156; p = 0.01967) was associated with increased parasite density in *P. falciparum*-infected patients. Furthermore, the rs5744292-G allele was significantly associated with higher parasitemia levels under the dominant model with an OR of 6.578. We also found heterozygous genotype CG for SNP rs187238 (OR = 0.452) and AG for SNP rs1293344 (OR = 0.492), to be associated with variations in malarial parasitemia, when compared with homozygous genotypes CC and AA, respectively. Under a recessive model of inheritance, rs360714-G allele showed a significant association with increased levels of parasitic density (OR = 17).

We also compared allele frequency for these SNPs in group 1+2+3 against group 4. Under a recessive mode of inheritance, we found that rs549908 G allele (OR = 0.290) and rs360729 T allele (OR = 0.272) were both significantly associated with increased level of parasitemia. No other association was observed between these two groups (Table 3).

Haplotype Association Analysis

Haplotype analysis was done between the healthy control group and *P. falciparum*-infected patients and the result is summarized in Table 4. Out of the three haplotypes identified, the occurrence frequency of two haplotypes, GT and AT were significant between these two groups. GT (rs544354 and rs549908) was found to be more frequent within the healthy controls (*f* = 0.586), compared to *P. falciparum*-infected patients (*f* = 0.516), (χ^2 = 4.352; p = 0.037). However, AT (rs544354 and rs549908) was more prevalent among the *P. falciparum*-infected patients (*f* = 0.248), compared to the healthy controls (*f* = 0.187), (χ^2 = 4.834; p = 0.028).

Similar haplotype analysis was done between *P. falciparum*-infected patients in group 2+3+4 and group 1 for parasitic density. We observed that the haplotype CAT (rs360728, rs360729 and rs549908) had significantly higher frequency (*f*=0.821) within the group 1 patients, compared to combined group 2+3+4 patients (*f* = 0.637), (χ^2 = 3.926; p = 0.048) (Table 5).

Genotype and Allele Frequency Analysis Based on Age

We also examined the relation of the 16 selected SNPs in the *IL-18* gene with the age of the *P. falciparum*-infected patients. For this analysis, the 250 patients with parasitemia were divided into different age groups. The genotypic distribution of patients in 1–9 years age group compared to the patient group ≥10 years of age is represented in Table 6. We observed rs544354 (OR = 0.407; 95% CI = 0.204–0.812; p = 0.008) and rs360714 (OR = 0.256; 95% CI = 0.119–0.554; p=0.001) to be significantly associated with age in *P. falciparum*-infected patients. Heterozygous rs544354-AG genotype was present in significantly higher frequency (*f* = 0.77) in age group 1–9 years, compared to patients in ≥10 years age group (*f* = 0.35). It was observed that both under dominant (OR = 0.146; 95% CI = 0.041–0.519; p <0.001) and recessive (OR = 0.794; 95% CI = 0.099–6.355; p <0.001) models of inheritance, rs544354-A allele occurrence was associated with the age of the infected patients. Similarly, for SNP rs360714, heterozygous-AG genotype showed a higher incidence (*f* = 0.61) in *P. falciparum*-infected patients in the 1–9 years age group compared to patients above 10 years of age (*f* = 0.19). In the dominant model, rs360714-G allele was also significantly associated with patient age (OR = 0.157; 95% CI = 0.058–0.428; p <0.001).

Further analysis was performed between the patient group 1–9 years age group compared to the patient group ≥41 years of age (Table 7). The results showed that rs5744292 A allele (OR = 9.456; 95% CI = 0.546–163.840; p = 0.037), rs544354 AG genotype (OR = 0.080; 95% CI = 0.020–0.330; p=0.0001) and rs360714 AG genotype (OR = 0.163; 95% CI = 0.051–0.517; p=0.001) are significantly associated with age in *P. falciparum*-infected patients. Heterozygous rs544354-AG genotype was present in significantly higher frequency (*f* = 0.77) in age group 1–9 years, compared to patients in ≥41 years age group (*f* = 0.22). Also, heterozygous rs360714-AG was present in a significantly higher frequency (*f* = 0.61) in age group 1–9 years, compared to patients in ≥41 years age group (*f* = 0.20)

Haplotype Analysis Between Different Age Groups

We performed a haplotype analysis between the different age groups within the *P. falciparum*-infected patients. Haplotype analysis between the different age groups within the *P. falciparum* infected patients identified two haplotypes GG (rs544354 and rs360727) with a significantly higher frequency among ≥10 years age group (*f* = 0.674) compared to 1–9 years age group (*f* = 0.491), (χ^2 =

Table 3 Genotype Distribution and Allele Frequency of *IL-18* Gene SNPs When Group 1+2+3 of Parasite Density Was Compared with Group 4

SNPs	Genotype/Allele	Group (1+2+3) n=90	Group (4) n=160	OR (95% C.I.)	χ^2	P-value*
rs1946518	GG	26 (28.9%)	52 (32.5%)	Ref		
	GT	46 (51.1%)	74 (46.25%)	0.804 (0.443–1.462)	0.51	0.474
	TT	18 (20%)	34 (21.25%)	0.944 (0.450–1.980)	0.02	0.879
	G	98(54.4%)	178 (55.6%)	0.953 (0.661–1.376)	0.06	0.798
	T	82 (45.6%)	142 (44.4%)			
	TT+GT.vs.GG			0.844 (0.480–1.482)	0.35	0.554
	TT.vs.GT+GG					
rs1946519	CC	28 (31.1%)	54 (33.75%)	Ref		
	AC	45 (25%)	74 (46.25%)	0.853 (0.474–1.535)	0.28	0.595
	AA	17 (18.9%)	32 (20%)	0.976 (0.464–2.055)	0	0.949
	C	101 (56.1%)	182 (56.9%)	0.969 (0.671–1.401)	0.03	0.868
	A	79 (43.9%)	138 (43.1%)			
	AA+AC.vs.CC			0.886 (0.510–1.542)	0.18	0.669
	AA.vs.AC+CC			0.932 (0.484–1.793)	0.05	0.831
rs187238	CC	50 (55.6%)	98 (61.25%)	Ref		
	CG	36 (40%)	45 (28.1%)	0.638 (0.366–1.111)	2.54	0.111
	GG	4 (44.4%)	14 (8.75%)	1.786 (0.559–5.709)	0.98	0.322
	C	136 (75.6%)	241 (76.75%)	0.936 (0.610–1.438)	0.09	0.763
	G	44 (24.4%)	73 (23.25%)			
	GG+CG.vs.CC			0.753 (0.445–1.274)	1.12	0.289
	GG.vs.CG.CC					
rs360728	CC	69 (76.7%)	128 (80%)	Ref		
	CG	19 (21.1%)	25 (15.6%)	0.709 (0.365–1.378)	1.03	0.309
	GG	2 (2.2%)	7 (4.4%)	1.887 (0.381–9.331)	0.62	0.429
	C	157 (87.2%)	281 (87.81%)	0.947 (0.546–1.644)	0.04	0.847
	G	23 (12.8%)	39 (12.19%)			
	GG+CG.vs.CC			0.821 (0.440–1.532)	0.38	0.535
	GG.vs.CG+GG			0.497 (0.101–2.444)	0.77	0.380

rs5744292					
AA	80 (88.9%)	137 (85.6%)	Ref		
AG	9 (10%)	17 (10.6%)	1.103(0.470–2.590)	0.05	0.821
GG	1 (1.1%)	6 (3.8%)	3.504 (0.42–29.63)	1.5	0.221
A	169 (93.9%)	291 (90.9%)	1.531 (0.75–3.14)	1.36	0.242
G	11 (6.1%)	29 (9.1%)			
GG+AG.vs.AA			1.343 (0.608–2.965)	0.54	0.464
GG.vs.AG+AA			0.288 (0.034–2.434)	1.47	0.224
rs360727					
GG	68 (75.6%)	128 (80%)	Ref		
AG	19 (21.1%)	26 (16.25%)	0.727 (0.376–1.407)	0.9	0.343
AA	3 (3.3%)	6 (3.75%)	1.062 (0.258–4.382)	0.01	0.933
G	155 (86.1%)	282 (88.1%)	0.835 (0.486–1.436)	0.42	0.514
A	25 (13.9%)	38 (11.9%)			
AA+AG.vs.GG			0.773 (0.417–1.433)	0.67	0.412
AA.vs.AG+AA			0.885 (0.216–3.627)	0.03	0.865
rs549908					
TT	54 (75.6%)	98 (61.3%)	Ref		
GT	33 (36.7%)	45 (28.1%)	0.751 (0.430–1.314)	1.01	0.315
GG	3 (3.3%)	17 (10.6%)	3.122 (0.876–11.136)	3.36	0.066
T	141 (78.3%)	241 (75.3%)	1.185 (0.766–1.833)	0.58	0.445
G	39 (43.3%)	79 (24.7%)			
GG+GT.vs.TT			0.949 (0.560–1.609)	0.04	0.845
GG.vs.GT+TT			0.290 (0.083–1.018)	4.16	**0.041**
rs11214101					
TT	89 (98.9%)	160 (100%)	Ref		
CT	1 (1.1%)	0 (0%)	0.186(0.007–4.611)	1.78	0.181
CC	0 (0%)	0 (0%)	0.558 (0.011–28.343)	Nan	1.000
T	179 (99.4%)	320 (100%)	0.187 (0.008–4.607)	1.78	0.481
C	1 (0.6%)	0 (0%)			
CC+CT.vs.TT			0.186 (0.007–4.611)	1.78	0.181
CC.vs.CT+TT			1.773 (0.035–90.137)	Nan	1.000
rs544354					
GG	46 (51.1%)	91 (56.9%)	Ref		
AG	40 (44.4%)	56 (35%)	0.708 (0.413–1.213)	1.59	0.207
AA	4 (4.4%)	13 (8.1%)	1.643 (0.507–5.322)	0.7	0.404
G	132 (73.3%)	238 (74.4%)	0.947 (0.626–1.435)	0.06	0.798
A	48 (26.7%)	82 (25.6%)			
AA+AG.vs.GG			0.793 (0.472–1.331)	0.77	0.379
AA.vs.AG+AA			0.526 (0.166–1.664)	1.23	0.267

(*Continued*)

Table 3 (Continued).

SNPs	Genotype/Allele	Group (1+2+3) n=90	Group (4) n=160	OR (95% C.I.)	χ^2	P-value*
rs360729	AA	53 (58.9%)	99 (61.9%)	Ref		
	AT	34 (37.8%)	43 (26.9%)	0.677 (0.387–1.185)	1.87	0.171
	TT	3 (3.3%)	18 (11.2%)	3.212 (0.905–11.403)	3.57	0.058
	A	140 (77.8%)	241 (75.3%)	1.147 (0.744–1.770)	0.39	0.534
	T	40 (22.2%)	79 (24.7%)			
	TT+AT.vs.AA			0.883 (0.521–1.496)	0.22	0.642
	TT.vs.AT+AA			0.272 (0.078–0.950)	4.69	**0.030**
rs5744260	GG	85 (94.4%)	154 (96.25%)	Ref		
	AG	5 (5.6%)	6 (3.75%)	0.662 (0.196–2.235)	0.45	0.504
	AA	0 (0%)	0 (0%)	0.553 (0.011–28.14)	Nan	1
	G	175 (97.2%)	314 (98.1%)	0.669 (0.201–2.223)	0.44	0.535
	A	5 (2.8%)	6 (1.9%)			
	AA+AG.vs.GG			0.662 (0.196–2.235)	0.45	0.505
	AA.vs.AG+GG			1.773 (0.04–90.14)	Nan	1
rs360714	AA	70 (77.8%)	123 (76.9%)	Ref		
	AG	19 (21.1%)	37 (23.1%)	1.108 (0.593–2.073)	0.1	0.747
	GG	1 (1.1%)	0 (0%)	0.190 (0.008–4.734)	1.74	0.186
	A	159 (88.3%)	283 (88.4%)	0.990 (0.560–1.750)	0	0.972
	G	21 (11.7%)	37 (11.6%)			
	GG+AG.vs.AA			1.053 (0.567–1.953)	0.03	0.870
	GG.vs.AG+AA					
rs7106524	GG	37 (41.1%)	60 (37.5%)	Ref		
	AG	38 (42.2%)	70 (43.75%)	1.136 (0.643–2.007)	0.19	0.660
	AA	15 (16.7%)	30 (13.75%)	1.233 (0.587–2.593)	0.31	0.579
	G	112 (62.2%)	190 (59.4%)	1.127 (0.775–1.640)	0.39	0.532
	A	68 (37.8%)	130 (40.6%)			
	AA+AG.vs.GG			1.164 (0.686–1.973)	0.32	0.573
	AA.vs.AG+AA			0.857 (0.438–1.714)	0.17	0.680

SNP	Genotype			OR (95% CI)		p-value
rs5744231	CC	87 (96.7%)	154 (96.3%)	Ref		
	CT	3 (3.3%)	5 (3.1%)	0.942 (0.220–4.035)	0.01	0.935
	TT	0 (0%)	1 (0.6%)	1.699 (0.068–42.16)	0.56	0.452
	C	177 (98.3%)	313 (97.8%)	1.319 (0.337–5.167)	0.16	1.000
	T	3 (1.7%)	7 (2.2%)			
	TT+CT.vs.CC			1.130 (0.276–4.631)	0.03	0.865
	TT.vs.CT+CC			0.587 (0.024–14.57)	0.56	0.452
rs360718	AA	50 (55.5%)	96 (60%)	Ref		
	AC	32 (35.6%)	48 (30%)	0.781 (0.445–1.372)	0.74	0.389
	CC	8 (8.9%)	16 (10%)	1.042 (0.417–2.601)	0.01	0.930
	A	132 (73.3%)	240 (75%)	0.917 (0.605–1.390)	0.17	0.681
	C	48 (26.7%)	80 (25%)			
	CC+AC.vs.AA			0.833(0.494–1.405)	0.47	0.493
	CC.vs.AC+AA			0.878 (0.360–2.140)	0.08	0.774
rs1293344	AA	50 (55.6%)	95 (59.4%)	Ref		
	AG	36 (40%)	49 (30.6%)	0.716 (0.413–1.242)	1.42	0.233
	GG	4 (4.44%)	16 (10%)	2.105 (0.668–6.635)	1.67	0.195
	A	136 (75.6%)	239 (74.7%)	1.048 (0.686–1.599)	0.05	0.829
	G	44 (24.4%)	81 (25.3%)			
	GG+AG.vs.AA			0.855 (0.507–1.441)	0.34	0.556
	GG.vs.AG+AA			0.419 (0.136–1.293)	2.42	0.120
rs5744260	GG	85 (94.4%)	154 (96.25%)	Ref		
	AG	5 (5.6%)	6 (3.75%)	0.662 (0.196–2.235)	0.45	0.504
	AA	0 (0%)	0 (0%)	0.553 (0.011–28.14)	Nan	1.000
	G	175 (97.2%)	314 (98.1%)	0.669 (0.201–2.223)	0.44	0.535
	A	5 (2.8%)	6 (1.9%)			
	AA+AG.vs.GG			0.662 (0.196–2.235)	0.45	0.505
	AA.vs.AG+GG			1.773 (0.04–90.14)	Nan	1.000

Note: *Bold indicates significance.

Table 4 Haplotype Analysis of *IL-18* Gene Polymorphisms Among *P. falciparum*-Infected Patients versus Healthy Control Subjects

Haplotype Block		Freq.	Plasmodium Infected Patients, Healthy Control Ratio Counts	Plasmodium Infected Patients, Healthy Control Frequencies	Chi Square	P value *
rs544354	rs549908					
G	T	0.547	258.1: 241.9, 234.3: 165.7	0.516, 0.586	4.352	**0.037**
G	G	0.224	111.9: 388.1, 89.7: 310.3	0.224, 0.224	0.000	0.990
A	T	0.221	123.9: 376.1, 74.7: 325.3	0.248, 0.187	4.834	**0.028**

Note: *Bold indicates significance.

Table 5 Haplotype Analysis of *IL-18* Gene Polymorphisms in Group 1 versus Group 2+3+4 of *P. falciparum*-Infected Patients

Haplotypes Block			Freq.	Group (2+3+4), Group (1) Ratio Counts	Group (2+3+4), Group (1) Frequencies	Chi Square	P value *
rs360728	rs360729	rs549908					
C	A	T	0.647	294.2: 167.8, 23.0: 5.0	0.637, 0.821	3.926	**0.048**
C	T	G	0.19	89.1: 372.9, 4.0: 24.0	0.193, 0.142	0.434	0.509
G	A	T	0.097	46.6: 415.4, 1.0: 27.0	0.101, 0.036	1.28	0.258
G	T	G	0.026	12.8: 449.2, 0.0: 28.0	0.028, 0.000	0.794	0.373
C	T	T	0.021	10.2: 451.8, 0.0: 28.0	0.022, 0.000	0.607	0.436
C	A	G	0.015	7.6: 454.4, 0.0: 28.0	0.016, 0.000	0.444	0.505

Note: *Bold indicates significance.

4.93; P = 0.026), and haplotypes AA (rs544354 and rs360727) with a significantly higher frequency among 1–9 years age group (*f* = 0.130) compared to in patients above 10 years of age (*f* = 0.045), (χ^2 = 4.984; P = 0.026) (Table 8).

Haplotype analysis was also done for the *IL-18* SNPs between the patients within the age groups 1–9 years and ≥41 years. Two significant haplotypes were identified for these age groups (Table 9). This included haplotype AAG (rs360729, rs360714 and rs7106524), present with increased frequency within the age group ≥41 years (*f* = 0.375) compared to patients within age group 1–9 years (*f* = 0.168), (χ^2 = 4.917; p = 0.027), while haplotype AGG (rs360729, rs360714 andrs7106524) was present with increased frequency among the patients in the age group 1–9 years (*f* = 0.278), compared to patients above 41 years of age (*f* = 0.103), (χ^2 = 5.687; p = 0.017).

Effect of IL-18 SNPs on Expression Levels in Luciferase Reporter Assay

Luciferase reporter assay was performed to investigate the effect of the SNPs in the promoter region of *IL-18* on its expression levels. Several DNA fragments of the *IL-18* promoter were cloned and transfected in HuH7 cells and the variations in the luciferase gene activity were monitored. Figure 2 shows the schematic representation of the influence of *IL-18* promoter segments, with the corresponding SNPs, on its transcriptional activity. Three different promoter fragments represented as; fragment 1 [from −795 to +28], fragment 2 [from −795 to −523] and fragment 3 [from −182 to +28] were analyzed. We found that fragment 1 exhibited high luciferase activity compared to fragments 2 and 3 (Figure 2A). Fragment 1 includes the two SNPs, rs1946518 and rs187238 that were studied here. As such, we generated three additional fragments that contain different haplotype combinations. Haplotype TG (rs1946518 and rs187238) showed the highest luciferase activity (Figure 2B).

The luciferase reporter assay was also conducted for different DNA segments of the 3′UTR region of the *IL-18* gene. Three different fragments of the 3′UTR *IL-18* region analyzed were represented as fragment 1 [from +20472 to +20984], fragment 2 [from +20500 to +20984] and fragment 3 [from +20472 to +20733]. Fragment 3 containing 2 SNPs, rs360728 and rs5744292, showed the highest level of luciferase activity (Figure 3A). As it showed the highest luciferase activity, fragment 3 was selected for haplotype expression analysis. Three different allelic combinations for SNPs rs360728 and rs5744292 were tested for their effect on luciferase expression. Haplotype CG (rs360728 and rs5744292) was found to have the highest luciferase activity (Figure 3B).

Table 6 Genotype Distribution and Allele Frequency of *IL-18* Gene SNPs When Age Group 1–9 Years Was Compared with Group 10 Years and Above

SNPs	Genotype/Allele	Group (1–9 Years) n=18	Group (10 Years and Above) n=232	OR (95% C.I.)	χ^2	P-value*
rs1946518	GG	3 (16.7%)	75(32.3%)	Ref		
	GT	12 (66.6%)	108(46.6%)	0.360 (0.098–1.320)	2.56	0.109
	TT	3 (16.7%)	49(21.1%)	0.653 (0.127–3.369)	0.26	0.608
	G	18 (50%)	258(55.6%)	0.798 (0.405–1.574)	0.42	0.514
	T	18 (50%)	206(44.4%)			
	TT+GT.vs.GG			0.419 (0.118–1.490)	1.91	0.167
	TT.vs.GT+GG			0.747 (0.208–2.684)	0.20	0.653
rs1946519	CC	3 (16.7%)	79 (34.05%)	Ref		
	AC	12 (66.7%)	107 (46.12%)	0.339 (0.092–1.240)	2.9	0.088
	AA	3 (16.6%)	46(19.83%)	0.582 (0.113–3.005)	0.43	0.513
	C	18 (50%)	265(57.1%)	0.751 (0.381–1.480)	0.69	0.406
	A	18 (50%)	199(42.9%)			
	AA+AC.vs.CC			0.387 (0.109–1.378)	2.29	0.130
	AA.vs.AC+CC			0.809 (0.225–2.911)	0.11	0.744
rs187238	CC	14(77.8%)	134(57.75%)	Ref		
	CG	4(22.2%)	77(33.2%)	2.011 (0.639–6.326)	1.48	0.224
	GG	0(0%)	21(9.05%)	4.636 (0.26–80.6)	2.17	0.141
	C	32(88.9%)	345(74.4%)	2.759 (0.956–7.966)	3.81	0.051
	G	4(11.1%)	119(25.6%)			
	GG+CG.vs.CC			2.560 (0.818–8.014)	2.77	0.095
	GG.vs.CG.CC			0.266 (0.015–4.568)	1.78	0.182
rs360728	CC	12(66.7%)	185(79.74%)	Ref		
	CG	5(27.8%)	39(16.81%)	0.506 (0.16–1.5)	1.52	0.216
	GG	1(5.5%)	8(3.45%)	0.519 (0.06–4.49)	0.37	0.544
	C	29(80.6%)	409(88.1%)	0.557 (0.23–1.33)	1.77	0.189
	G	7(19.4%)	55(11.9%)			
	GG+CG.vs.CC			0.508 (0.18–1.4)	1.71	0.191
	GG.vs.CG+GG			1.647 (0.19–13.9)	0.21	0.643

(*Continued*)

Table 6 (Continued).

SNPs	Genotype/Allele	Group (1–9 Years) n=18	Group (10 Years and Above) n=232	OR (95% C.I.)	χ^2	P-value*
rs5744292	AA	18(100%)	199(85.8%)	Ref	2.33	0.126
	AG	0(0%)	26(11.2%)	4.915 (0.28–83.96)	0.63	0.426
	GG	0(0%)	7(3.0%)	1.391 (0.07–25.33)	3.37	0.101
	A	36(100%)	424(91.4%)	6.965 (0.42–115.6)		
	G	0(0%)	40(8.6%)			
	GG+AG.vs.AA			6.213 (0.36–105.5)	2.95	0.085
	GG.vs.AG+AA			0.813 (0.04–14.8)	0.56	0.454
rs360727	GG	12(66.7%)	184(79.31%)	Ref	1.39	0.238
	AG	5(27.8%)	40(17.24%)	0.522 (0.174–1.564)	0.36	0.548
	AA	1(5.5%)	8(3.45%)	0.522 (0.060–4.521)	1.65	0.195
	G	29(80.6%)	408(87.93%)	0.569 (0.238–1.359)		
	A	7(19.4%)	56(12.07%)			
	AA+AG.vs.GG			0.522 (0.186–1.462)	1.58	0.209
	AA.vs.AG+AA			1.647 (0.194–13.951)	0.21	0.643
rs549908	TT	13 (72.2%)	139(59.9%)	Ref	0.33	0.566
	GT	5(27.8%)	73(31.5%)	1.365 (0.469–3.979)	1.85	0.173
	GG	0(0%)	20(8.6%)	3.968 (0.227–69.317)	2.03	0.154
	T	31(86.1%)	351(75.6%)	1.996 (0.758–5.255)		
	G	5(13.9%)	113(24.4%)			
	GG+GT.vs.TT			1.740 (0.600–5.043)	1.06	0.302
	GG.vs.GT+TT			0.280 (0.016–4.821)	1.69	0.194
rs11214101	TT	18(100%)	231(99.6%)	Ref	0.08	0.780
	CT	0(0%)	1(0.4%)	0.240 (0.009–6.094)	Nan	1.000
	CC	0(0%)	0(0%)	0.080 (0.002–4.144)		1.002
	T	36(100%)	463(99.8%)	0.236 (0.009–5.903)	0.08	
	C	0(0%)	1(0.2%)			
	CC+CT.vs.TT			0.240 (0.009–6.064)	0.08	0.780
	CC.vs.CT+TT			12.568 (0.242–651.669)	Nan	1

			OR (95% CI)		p-value
rs544354					
GG	3 (16.66%)	134(57.76%)	Ref		
AG	14 (77.78%)	82(35.34%)	0.131 (0.037–0.470)	12.82	**<0.001**
AA	1 (5.56%)	16(6.90%)	0.358 (0.035–3.652)	0.82	0.366
G	20 (55.56%)	350(75.43%)	0.407 (0.204–0.812)	6.86	**0.008**
A	16(44.44%)	114(24.57%)			
AA+AG.vs.GG			0.146 (0.041–0.519)	11.39	**<0.001**
AA.vs.AG+AA			0.794 (0.099–6.355)	0.05	**<0.001**
rs360729					
AA	13(72.2%)	139(59.9%)	Ref		
AT	4(22.2%)	73(31.5%)	1.707 (0.537–5.422)	0.84	0.359
TT	1(5.6%)	20(8.6%)	1.871 (0.23–15.08)	0.36	0.550
A	30(83.3%)	351(75.6%)	1.610 (0.653–3.966)	1.09	0.296
T	6(16.7%)	113(24.4%)			
TT+AT.vs.AA			1.740 (0.600–5.043)	1.06	0.302
TT.vs.AT+AA			0.624 (0.079–4.933)	0.2	0.651
rs360714					
AA	7 (38.89%)	186(80.17%)	Ref		
AG	11 (61.11%)	45 (19.40%)	0.154 (0.057–0.419)	16.6	**0.001**
GG	0 (0%)	1 (0.43%)	0.121 (0.005–3.215)	0.04	0.846
A	25 (69.44%)	417(89.87%)	0.256 (0.119–0.554)	13.59	**0.001**
G	11 (30.56%)	47(10.13%)			
GG+AG.vs.AA			0.157 (0.058–0.428)	16.17	**<0.001**
GG.vs.AG+AA			4.171 (0.164–106.0)	0.08	0.780
rs7106524					
GG	6(33.3%)	91 (39.2%)	Ref		
AG	9(50%)	99(42.7%)	0.725 (0.248–2.118)	0.35	0.555
AA	3(16.7%)	42 (18.1%)	0.923 (0.220–3.870)	0.01	0.912
G	21(58.3%)	281(60.6%)	0.912 (0.458–1.815)	0.07	0.792
A	15(41.7%)	183(39.4%)			
AA+AG.vs.GG			0.775 (0.281–2.137)	0.24	0.621
AA.vs.AG+AA			0.905 (0.251–3.267)	0.02	0.878
rs5744231					
CC	18 (100%)	223 (96.12%)	Ref		
CT	0 (0%)	8 (3.45%)	1.407 (0.078–25.35)	0.64	0.422
TT	0 (0%)	1 (0.43%)	0.248 (0.010–6.312)	0.08	0.776
C	36 (100%)	454 (97.8%)	1.686 (0.097–29.36)	0.79	1.000
T	0 (0%)	10 (2.2%)			
TT+CT.vs.CC			1.573 (0.09–28.103)	0.72	0.394
TT.vs.CT+CC			4.171 (0.16–106.02)	0.08	0.780

(*Continued*)

Table 6 (Continued).

SNPs	Genotype/Allele	Group (1–9 Years) n=18	Group (10 Years and Above) n=232	OR (95% C.I.)	χ^2	P-value*
rs360718	AA	13 (72.2%)	133 (57.33%)	Ref		
	AC	5 (27.8%)	75 (32.33%)	1.466 (0.503–4.272)	0.5	0.480
	CC	0 (0%)	24 (10.34%)	4.955 (0.28–86.122)	2.31	0.128
	A	31 (86.1%)	341 (73.5%)	2.236 (0.850–5.881)	2.79	0.094
	C	5 (13.9%)	123 (26.5%)			
	CC+AC.vs.AA			1.935 (0.668–5.607)	1.53	0.216
	CC.vs.AC+AA			0.230 (0.013–3.937)	2.06	0.151
rs1293344	AA	12 (66.7%)	133 (57.33%)	Ref		
	AG	6 (33.3%)	79 (34.05%)	1.188 (0.429–3.290)	0.11	0.740
	GG	0 (0%)	20 (8.62%)	3.839 (0.21–67.34)	1.78	0.181
	A	30 (83.3%)	345 (74.4%)	1.725 (0.70–4.346)	1.44	0.230
	G	6 (16.7%)	119 (25.6%)			
	GG+AG.vs.AA			1.489 (0.54–4.10)	0.6	0.439
	GG.vs.AG+AA			0.280 (0.02–4.82)	1.69	0.194

Note: *Bold indicates significance.

Discussion

IL-18, an important immune response mediator that has been reported to be involved in the course of malarial infection.[20–24] IL-18 is known as a potent proinflammatory cytokine, and its physiological levels have been reported to increase at different stages of *P. falciparum* infection.[31] Information about the influence of genetic variations within the *IL-18* gene on parasitemia caused by *P. falciparum* is scarce. In this study, we examined the plausible role and influence of polymorphisms within the *IL-18* gene on parasitemia levels in *P. falciparum*-infected patients. We found SNP rs5744292 to be significantly associated with an increased risk of *P. falciparum* infection when compared to healthy controls. It was observed that the rs5744292-G allele occurred more frequently in infected individuals than in the control group. rs5744292 has been implicated in several diseases such as cardiovascular diseases[32,33] and coronary atherosclerosis.[34] It has also been reported that a decrease in IL-18 concentration is associated with the G allele of rs5744292.[25] We also identified another SNP, rs544354-A allele, to be significantly associated with an increased risk of parasitemia in infected patients. Further, individuals with homozygous AA genotype were found to have an elevated risk of parasitemia caused by *P. falciparum*. There is very little information available about the clinical impact of the SNP rs544354 which is located in the 3′UTR of the *IL-18* gene.[35] Another polymorphism found to be significantly associated with parasitemia in the infected patients was the rs360714-*GG* homozygote, present in the intron region of the *IL-18* gene. However, this result needs to be interpreted with caution as the number of individuals in study population carrying GG genotype at the rs360714 SNP were very few.

SNP rs7106524, also located in the intron region of the *IL-18* gene, has been investigated for its role in several clinical conditions such as those involving liver transplant.[36,37] A previous study by He et al (2010) found an association between polymorphism rs7106524 with IL-18 levels, where the minor allele genotype (AA) was found to be associated with the highest concentration of serum IL-18.[38] We found rs7106524 heterozygous GA genotype to be associated with an increased risk of *P. falciparum* parasitemia in comparison to the other two homozygous genotypes. Possibly, moderate levels of IL-18 linked to rs7106524-GA genotype may have a functional consequence in increasing the risk of malarial

Table 7 Genotype Distribution and Allele Frequency of *IL-18* Gene SNPs When Age Group 1–9 Years Was Compared with Group 41 Years and Above

SNPs	Genotype/ Allele Distribution	Group (1–9 Years) n=18	Group(41 Years and Above) n=54	OR (95% C.I.)	χ²	P-value*
rs1946518	GG	3 (16.7%)	20 (37.03%)	Ref	3.76	0.052
	GT	12 (66.6%)	21 (38.9%)	0.263 (0.064–1.070)	0.24	0.627
	TT	3 (16.7%)	13 (24.07%)	0.650 (0.113–3.726)	0.46	0.498
	G	18 (50%)	61 (56.5%)	0.770 (0.362–1.641)		
	T	18(50%)	47 (43.5%)			
	TT+GT.vs.GG			0.340 (0.088–1.321)	2.58	0.108
	TT.vs.GT+GG			0.631 (0.157–2.527)	0.43	0.512
rs1946519	CC	3 (16.7%)	18 (33.33%)	Ref	2.89	0.089
	AC	12 (66.6%)	22 (40.74%)	0.306 (0.075–1.252)	0.08	0.777
	AA	3 (16.7%)	14 (25.92%)	0.778 (0.136–4.458)	0.15	0.699
	C	18 (50%)	58 (53.7%)	0.862 (0.405–1.834)		
	A	18(50%)	50 (46.3%)			
	AA+AC.vs.CC			0.400 (0.102–1.563)	1.82	0.177
	AA.vs.AC+CC			0.571 (0.144–2.274)	0.64	0.423
rs187238	CC	14 (77.8%)	37 (68.51%)	Ref	0.1	0.750
	CG	4 (22.2%)	13 (24.07%)	1.230 (0.342–4.415)	1.47	0.224
	GG	0 (0%)	4 (7.4%)	3.480 (0.176–68.785)	1.31	0.252
	C	32 (88.9%)	87 (80.6%)	1.931 (0.615–6.059)		
	G	4 (11.1%)	21 (19.4%)			
	GG+CG.vs.CC			1.608 (0.460–5.617)	0.56	0.454
	GG.vs.CG.CC			0.303 (0.016–5.911)	1.41	0.234
rs360728	CC	12 (66.7%)	42 (77.8%)	Ref	0.78	0.376
	CG	5 (27.7%)	10 (18.5%)	0.571 (0.164–1.996)	0.2	0.655
	GG	1 (5.6%)	2 (3.7%)	0.571 (0.048–6.856)	0.91	0.339
	C	29 (80.5%)	94 (87.04%)	0.617 (0.227–1.674)		
	G	7 (19.4%)	14 (12.96%)			
	GG+CG.vs.CC			0.571(0.177–1.844)	0.89	0.345
	GG.vs.CG+GG			1.529 (0.130–17.939)	0.12	0.733

(*Continued*)

Table 7 (Continued).

SNPs	Genotype/ Allele Distribution	Group (1–9 Years) n=18	Group(41 Years and Above) n=54	OR (95% C.I.)	χ^2	P-value*
rs5744292	AA	18 (100%)	44 (81.5%)	Ref		
	AG	0 (0%)	8 (14.8%)	7.067(0.388–128.862)	3.13	0.077
	GG	0 (0%)	2 (3.7%)	2.079 (0.095–45.427)	0.81	0.368
	A	36 (100%)	96 (88.9%)	9.456 (0.546–163.840)	4.36	**0.037**
	G	0 (0%)	12 (11.1%)			
	GG+AG.vs.AA			8.730 (0.486–156.833)	3.87	**0.049**
	GG.vs.AG+AA			0.568 (0.026–12.377)	0.69	0.407
rs360727	GG	12 (66.7%)	44 (81.5%)	Ref		
	AG	5 (27.7%)	8 (14.8%)	0.436 (0.121–1.580)	1.65	0.199
	AA	1 (5.6%)	2 (3.7%)	0.545 (0.06–6.539)	0.23	0.627
	G	29 (80.5%)	96 (88.9%)	0.518 (0.187–1.437)	1.64	0.254
	A	7 (19.4%)	12 (11.1%)			
	AA+AG.vs.GG			0.455 (0.137–1.504)	1.71	0.190
	AA.vs.AG+AA			1.529 (0.130–17.939)	0.12	0.733
rs549908	TT	13 (72.2%)	39 (72.2%)	Ref		
	GT	5 (27.8%)	10 (18.5%)	0.667 (0.192–2.312)	0.41	0.521
	GG	0 (0%)	5 (9.3%)	3.759 (0.195–72.570)	1.62	0.203
	T	31 (86.1%)	88 (81.5%)	1.409 (0.487–4.075)	0.4	0.525
	G	5 (13.9%)	20 (18.5%)			
	GG+GT.vs.TT			1.00 (0.304–3.290)	0	1.000
	GG.vs.GT+TT			0.243 (0.013–4.619)	1.79	0.180
rs11214101	TT	18 (100%)	54 (100%)	Ref		
	CT	0 (0%)	0 (0%)	0.339 (0.007–17.722)	Nan	1.000
	CC	0 (0%)	0 (0%)	0.339 (0.007–17.722)	Nan	1.000
	T	36 (100%)	108 (100%)	0.336 (0.007–17.260)	Nan	1.000
	C	0 (0%)	0 (0%)			
	CC+CT.vs.TT			0.339 (0.007–17.722)	Nan	1.000
	CC.vs.CT+TT			2.946 (0.056–153.803)	Nan	1.000

rs544354	GG	Ref		0.0001
	AG	0.080 (0.020–0.330)	15.21	0.957
	AA	0.938 (0.087–10.048)	0	0.102
	G	0.526 (0.242–1.144)	2.67	
	A			
	AA+AG.vs.GG	0.138 (0.036–0.532)	9.8	0.001
	AA.vs.AG+AA	0.259(0.031–2.179)	1.75	0.185
rs360729	AA	Ref		
	AT	0.833 (0.223–3.115)	0.07	0.786
	TT	1.667 (0.178–15.607)	0.2	0.651
	A	1.136 (0.417–3.095)	0.06	0.802
	T			
	TT+AT.vs.AA	1.00 (0.304–3.290)	0	1.000
	TT.vs.AT+AA	0.576 (0.063–5.291)	0.24	0.622
rs5744260	GG	Ref		
	AG	0.308 (0.040–2.363)	1.41	0.234
	AA	0.314 (0.006–16.466)	Nan	1.000
	G	0.321 (0.044–2.365)	1.37	0.260
	A			
	AA+AG.vs.GG	0.308 (0.040–2.363)	1.41	0.234
	AA.vs.AG+AA	2.946 (0.056–153.803)	4	1.000
rs360714	AA	Ref		
	AG	0.163 (0.051–0.517)	10.56	0.001
	GG	0.172 (0.003–9.374)	Nan	1.000
	A	0.258 (0.100–0.663)	8.66	0.003
	G			
	GG+AG.vs.AA	0.163 (0.051–0.517)	10.56	0.001
	GG.vs.AG+AA	2.946 (0.056–153.803)	Nan	1.000
rs7106524	GG	Ref		
	AG	0.667 (0.204–2.178)	0.45	0.500
	AA	0.696 (0.140–3.454)	0.2	0.565
	G	0.791 (0.366–1.709)	0.36	0.550
	A			
	AA+AG.vs.GG	0.674 (0.220–2.063)	0.48	0.487
	AA.vs.AG+AA	1.150 (0.270–4.899)	0.04	0.850

Note: The percentage columns between the genotype and OR columns read as follows for each genotype:

rs544354: GG 3 (16.7%) / 32 (59.3%); AG 14 (77.8%) / 12 (22.2%); AA 1 (5.6%) / 10 (18.5%); G 20 (55.6%) / 76 (70.4%); A 16 (44.4%) / 32 (29.6%)

rs360729: AA 13 (72.2%) / 39 (72.2%); AT 4 (22.2%) / 10 (18.5%); TT 1 (5.6%) / 5 (9.3%); A 30 (83.3%) / 88 (81.5%); T 6 (16.7%) / 20 (18.5%)

rs5744260: GG 16 (88.9%) / 52 (96.3%); AG 2 (11.1%) / 2 (3.7%); AA 0 (0%) / 0 (0%); G 34 (94.4%) / 106 (98.1%); A 2 (5.6%) / 2 (1.9%)

rs360714: AA 7 (38.9%) / 43 (79.6%); AG 11 (61.1%) / 11 (20.4%); GG 0 (0%) / 0 (0%); A 25 (69.4%) / 97 (89.8%); G 11 (30.6%) / 11 (10.2%)

rs7106524: GG 6 (33.3%) / 23 (42.6%); AG 9 (50%) / 23 (42.6%); AA 3 (16.7%) / 8 (14.8%); G 21 (58.3%) / 69 (63.9%); A 15 (41.7%) / 39 (36.1%)

(Continued)

Table 7 (Continued).

SNPs	Genotype/ Allele Distribution	Group (1–9 Years) n=18	Group(41 Years and Above) n=54	OR (95% C.I.)	χ^2	P-value*
rs5744231	CC	18 (100%)	53 (98.1%)	Ref		
	CT	0 (0%)	1 (1.9%)	1.037 (0.040–26.594)	0.34	0.560
	TT	0 (0%)	0 (0%)	0.346 (0.007–18.056)	Nan	1
	C	36 (100%)	107 (99.07%)	1.019 (0.041–25.559)	0.34	1.044
	T	0 (0%)	1 (0.93%)			
	TT+CT.vs.CC			1.037 (0.040–26.594)	0.34	0.560
	TT.vs.CT+CC			2.96 (0.056–153.803)	Nan	1.000
rs360718	AA	13 (72.2%)	36 (66.7%)	Ref		
	AC	5 (27.8%)	13 (24.0%)	0.939 (0.280–3.151)	0.01	0.918
	CC	0 (0%)	5 (9.3%)	4.068 (0.210–78.641)	1.75	0.186
	A	31 (86.1%)	85 (78.7%)	1.678 (0.587–4.799)	0.95	0.330
	C	5 (13.9%)	23 (21.3%)			
	CC+AC.vs.AA			1.300 (0.401–4.215)	0.19	0.661
	CC.vs.AC+AA			0.243 (0.013–4.619)	1.79	0.180
rs1293344	AA	12 (66.7%)	37 (68.5%)	Ref		
	AG	6 (33.3%)	12 (22.2%)	0.649 (0.200–2.104)	0.52	0.469
	GG	0 (0%)	5 (9.3%)	3.667 (0.189–71.113)	1.57	0.209
	A	30 (83.3%)	86 (79.6%)	1.279 (0.473–3.455)	0.24	0.626
	G	6 (16.7%)	22 (20.4%)			
	GG+AG.vs.AA			0.919 (0.295–2.861)	0.02	0.883
	GG.vs.AG+AA			0.243 (0.013–4.619)	1.79	0.180

Note: *Bold indicates significance.

Table 8 Haplotype Analysis for *IL-18* Gene Polymorphisms Between Group 1 (1–9 Years) Was Compared to 10 Years and Above

Haplotype		Freq.	Case, Control Ratio Counts	Case, Control Frequencies	Chi Square	P-value *
rs544354	rs360727					
G	G	0.66	292.5: 141.5, 17.7: 18.3	0.674, 0.491	4.93	**0.026**
A	G	0.21	87.5: 346.5, 11.3: 24.7	0.202, 0.314	2.53	0.112
G	A	0.078	34.5: 399.5, 2.3: 33.7	0.080, 0.064	0.11	0.740
A	A	0.051	19.5: 414.5, 4.7: 31.3	0.045, 0.130	4.984	**0.026**

Note: *Bold indicates significance.

Table 9 Haplotype Analysis for *IL-18* Gene Polymorphisms Between Groups (1–9 Years) Was Compared to 41 Years and Above

| Haplotype			Freq.	Case, Control Ratio Counts	Case, Control Frequencies	Chi Square	P-value *
rs360729	rs360714	rs7106524					
A	A	A	0.366	27.8: 50.2, 13.9: 22.1	0.356, 0.387	0.105	0.746
A	A	G	0.31	29.2: 48.8, 6.1: 29.9	0.375, 0.168	4.917	**0.027**
A	G	G	0.158	8.0: 70.0, 10.0: 26.0	0.103, 0.278	5.687	**0.017**
T	A	G	0.146	11.8: 66.2, 4.9: 31.1	0.151, 0.137	0.036	0.850
T	A	A	0.012	1.2: 76.8, 0.1: 35.9	0.016, 0.002	0.442	0.506

Note: *Bold indicates significance.
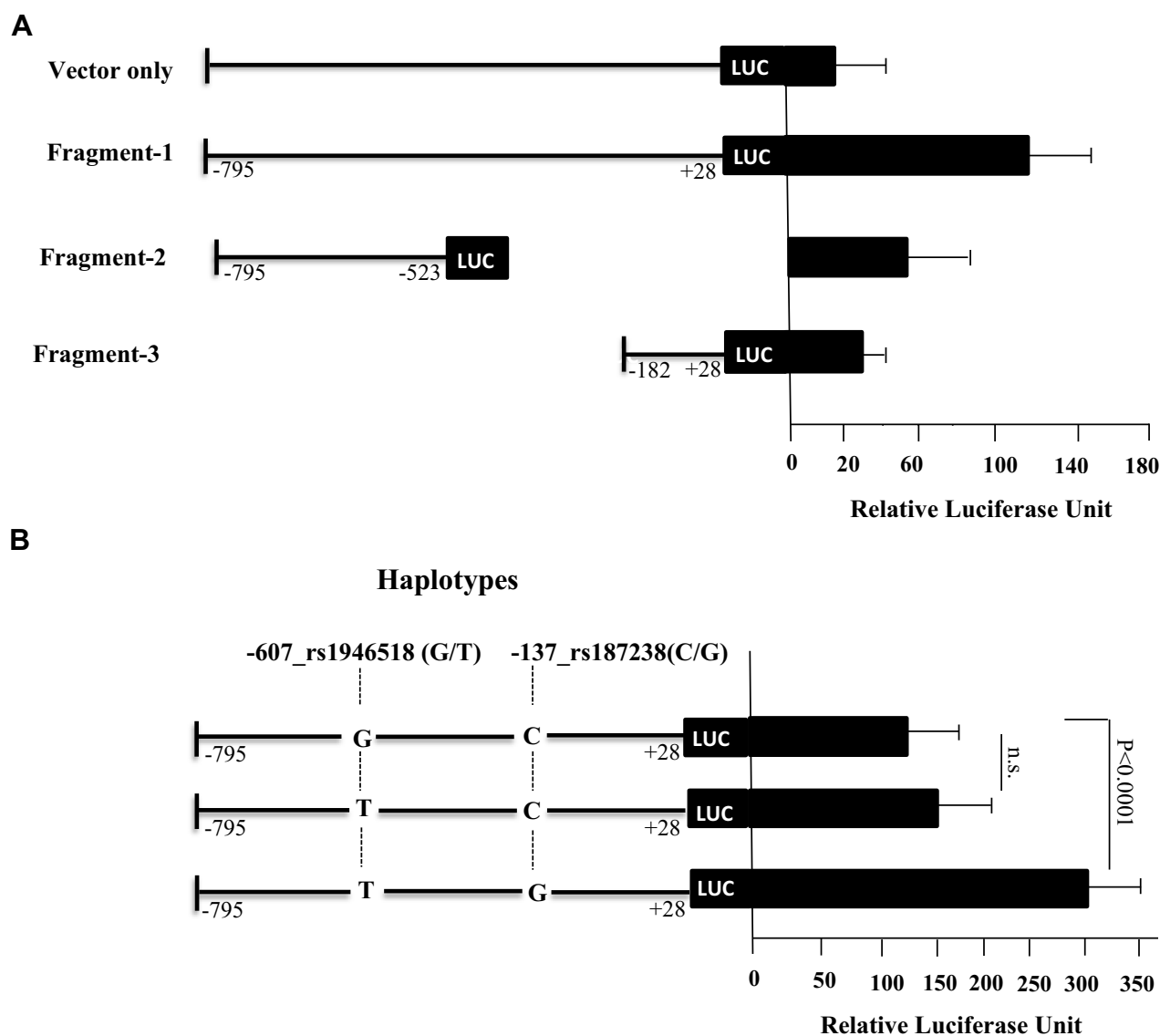
Figure 2 Luciferase activity assay for the fragments containing IL-18 promoter. (**A**) nested deletions of this region were generated, cloned into pGL3 luciferase vector and transfected in HuH7 cell line for 24 h. (**B**) As fragment 1 showed the highest luciferase activity, it was further tested to evaluate the effect of the SNPs included in this fragment on the expression activity. Cells were harvested and tested for luciferase activity after normalization using renilla luciferase enzyme. Each experiment was performed in triplicates. Values represent mean ±SD.

parasitemia and such a conclusion needs to be investigated further.

Haplotype analysis also revealed 2 haplotypes GT and AT (rs544354 and rs549908) that show significant differences between the controls and the infected patients. Haplotype GT was found more frequently in the controls whereas haplotype AT was found more frequently in the infected patient group. Haplotype AT includes rs544354 risk allele A whereas haplotype GT contains rs544354 allele G at the corresponding position. This haplotype analysis confirms the association of polymorphism rs544354 with an increased risk of *P. falciparum* infection.

We also analyzed the influence of these SNPs in the *IL-18* gene on the extent of parasitemia among the *P. falciparum*-infected patients and found the rs5744292-*G* allele to be significantly associated with increased parasite density in patients. Polymorphisms within the cytokine genes have been reported to be associated with various degrees of parasitemia. Sortica et al (2013) identified variations in IL-6 and IL-12B to be associated with the levels of parasitemia in *P. vivax*-infected Brazilian population.[39] Additionally, rs5744292 *G*-allele has been reported to be associated with downregulation of IL-18 levels.[25,32] The cellular/molecular mechanism through which rs5744292-*G*

A

Haplotypes

B
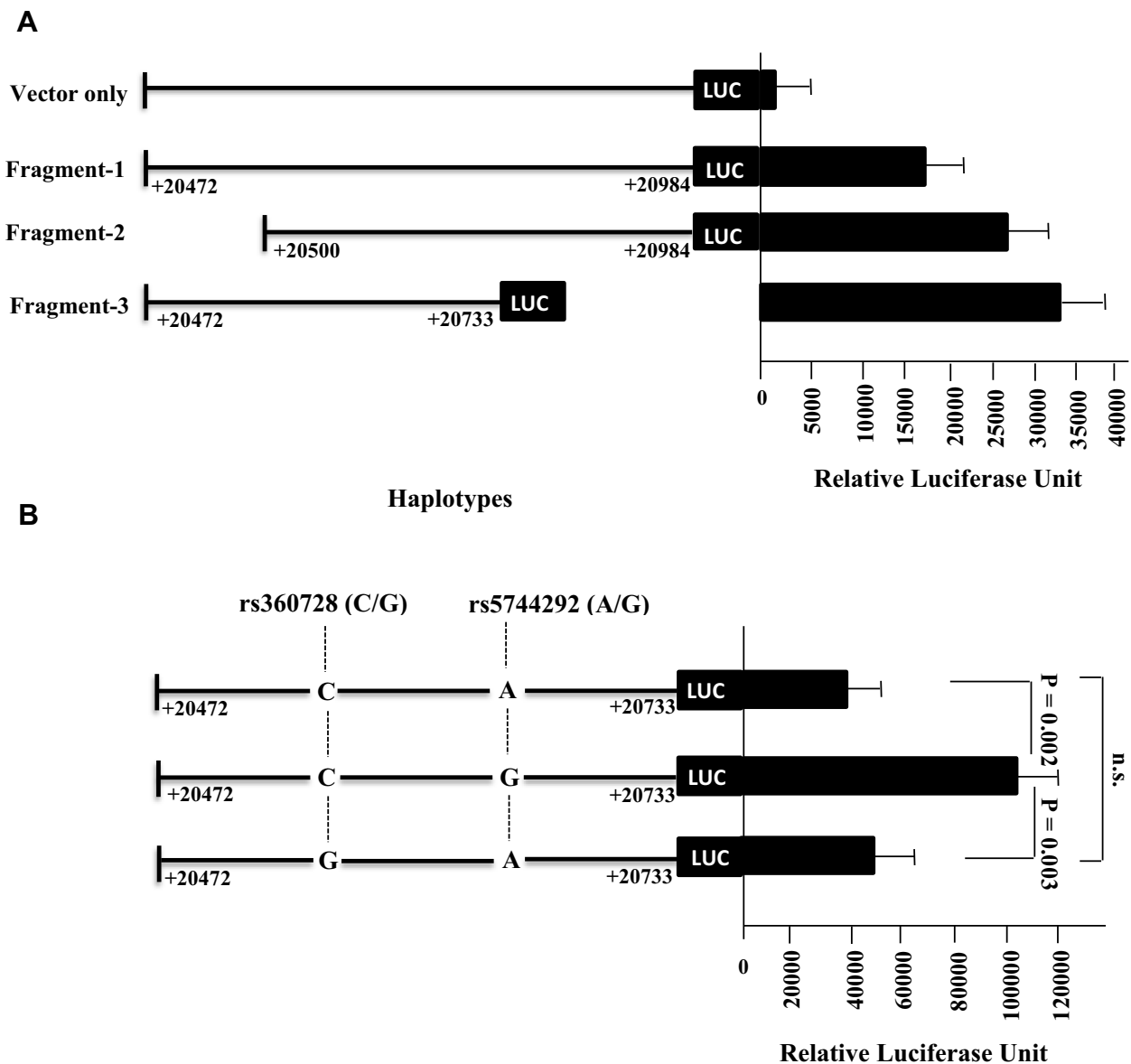
Figure 3 Luciferase activity assay for the fragments containing IL-18 3' UTR. (**A**) nested deletions of this region were generated, cloned into pGL3 luciferase vector and transfected in HuH7 cell line for 24 h. (**B**) As fragment 3 showed the highest luciferase activity, it was further tested to evaluate the effect of the SNPs included in this fragment on the expression activity. Cells were harvested and tested for luciferase activity after normalization using renilla luciferase enzyme. Each experiment was performed in triplicates. Values represent mean ±SD.

allele contributes to an increase in parasitemia in *P. falciparum*-infected patients is unclear and needs further investigation. Three more SNPs, rs187238, rs360714 and rs1293344, were also found to be significantly associated with varying parasitemia levels in our study group. We observed that rs187238-CG, rs360714-GG and rs1293344-AG genotypes were found to be associated with protection from a higher degree of parasitemia. SNP rs187238, located in the promoter region of *IL-18* gene, has been

previously associated with hepatitis C and type 1 diabetes.[40,41] Anyona et al (2011) observed that rs187238-C allele correlated with decreased IL-18 levels.[23] The authors of this study suggested that this G to C substitution destroys a histone 4 transcription factor-1 (H4TF-1) nuclear factor-binding site, resulting in lower promoter activity with subsequent downregulation of IL-18 levels. Based on these results, it can be inferred that the protective effect of rs187238 heterozygote variants CG

may be due to its regulation of IL-18 levels thereby controlling parasitemia.

Balavarca et al (2014) found that polymorphism rs1293344, located in the 5′ flanking region of the *IL-18* gene, is associated with improved overall survival of colorectal cancer.[42] Also, no significant association was found between polymorphism at rs1293344 and the changes in the serum concentration of IL-18.[43] In this study, we found rs1293344-AG genotype to be correlated with improved disease outcomes by protecting against increased parasitemia in *P. falciparum*-infected patients.

Our analysis also identified several haplotypes with differential frequencies among infected patients with varying degrees of parasitemia. A comparison of patients with the lowest degree of parasitemia with those that had a higher degree of parasitemia revealed one significant haplotype. The haplotype CAT exhibited a higher frequency in group 1 parasitemia patients than the combined group 2+3 +4 parasitemia patients. The mechanism through which this haplotype could affect the course of infection and/or expression of IL-18 requires further investigation.

Age is regarded as one of the crucial factors affecting the susceptibility and severity of the malarial infection.[44] It has been observed to correlate with protective immunity in malaria-endemic areas.[45] When compared to adolescents and adults, young children have been reported to be more susceptible to malarial infection, usually with severe complications that may be fatal if untreated.[8,45,46] Due to the development of acquired immunity, adults are protected from severe malaria.[46,47] In this study, we investigated the influence of the selected SNPs in the *IL-18* gene within the different age groups of the *P. falciparum*-infected patients. On comparing genotypic distribution among patients in the 1–9 years group with patients in age group ≥10 years, 2 SNPs- rs544354 and rs360714 were found to show a significant association with the latter group. We found that increased frequency of rs544354-A allele was predictive of parasitemia in the *P. falciparum*-infected patients included in the 1–9 years age group. Additionally, the A allele had a similar association in both dominant and recessive modes of inheritance as well as heterozygotic AG genotype. Similarly, a higher prevalence of the rs360714-G allele was associated with an increased risk of parasitemia in the patients of age group 1–9 years. Our results are consistent with the observation in the Kenyan population, where parasitemia was found to be strongly dependent on the age factor, with children under 5 years exhibiting the highest malarial parasitemia levels.[8] Further,

another study has reported overall higher levels of parasitemia in children ≤9 years.[48] IL-18 levels have also been reported to exhibit an age-related pattern in malarial patients.[49,50] The functional mechanism(s) of these *IL-18* SNPs must be studied to elucidate the role of these polymorphisms in determining age-related susceptibility to parasitemia.

We also performed a haplotype analysis between different age groups within the *P. falciparum*-infected patients. Single haplotype CA was found to be differentially distributed between the younger and older age groups. CA occurred more frequently within the 1–5 year age groups than in the older parasitemia patients. SNP rs187238 is positioned in the promoter region of the *IL-18* gene and its C allele has been reported to correlate with decreased concentration of IL-18.[23] The haplotype analysis among the parasitemia patients within the age groups 1–9 years and ≥ 41 years revealed the presence of two haplotypes AAG and AGG of SNPs rs360729, 360714 and rs7106524, respectively, which showed differential distribution between these groups. AAG occurred more frequently in older patients whereas haplotype AGG was found to occur at a higher frequency among the 1–9 years age group. Although rs360714 is located in the intron region of *IL-18* gene, the two haplotypes, AAG and AGG differ only with regards to rs360714 A or G allele. This suggest that rs360714 does have some functional impact in different age groups, a conclusion that needs further testing to confirm its biological/physiological importance.

The functional activity of the SNPs in the promoter and 3′UTR of the *IL-18* gene was analyzed by luciferase reporter assay. Two SNPs, rs1946518 and rs187238, of the promoter region within the *IL-18* gene were found to modulate the transcriptional activity in the vector system. Our results are in agreement with the previous studies that have reported an association of promoter polymorphism with IL-18 expression. A study investigating patients with sarcoidosis analyzed the promoter activity by luciferase assay and found polymorphisms rs1946519 and rs1946518 to be significantly associated with serum levels of IL-18.[51] Liang et al (2005) also reported the association of rs187238, among several other SNPs, with the transcriptional activity in the reporter assay system.[52] Another study examining the functional activity of *IL-18* promoter region found polymorphisms rs1946518 and rs187238 to be associated with the transcriptional activity.[53] Similarly, the SNPs in the potential regulatory 3′UTR region have been shown to

affect the expression levels of IL-18. Previous studies have reported that the 3′UTR region of the *IL-18* gene contains regulatory elements that may be responsible for mRNA stability and translation rate.[41,54] Polymorphism rs5744292 has been previously found to influence the IL-18 expression in various clinical conditions such as coronary artery disease patients.[25,32] Although not much is known about the polymorphism rs360728 in relation to IL-18 expression, we found rs360728-C/rs5744292-G haplotype to be associated with enhanced luciferase activity. This was in contrast to the previous report that linked rs5744292-G containing haplotypes with decreased transcriptional activity,[54] as well as our observation that rs5744292-G allele is linked to upregulation of IL-18 expression. These variations may be attributed to the fact that in-vitro studies using isolated fragments of the regulating regions may not be able to correctly reflect the functional activity *in-vivo*. Different elements including both cis- and trans-acting can influence the folding of the regulatory regions and subsequently their transcriptional activity. These results need to be confirmed *in-vivo* to clarify the role of these SNPs in modulating IL-18 expression.

In conclusion, this study has identified several individual polymorphisms and haplotypes within the pro-inflammatory *IL-18* gene and revealed their association with the risk and extent of parasitemia in *P. falciparum*-infected patients. This is the first time such a detailed analysis of *IL-18* gene polymorphism concerning malaria has been reported in Saudi Arabia, which in turn provides important insights into the role of population genetics in malaria.

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Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis, and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

Disclosure

The authors have nothing to declare with respect to the research, authorship, and/or publication of this article.

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