antibodies as described (Troye-Blomberg M 1983). For detection of IgG, IgG2, IgG3 and IgE antibodies, following antibodies or monoclonal antibodies were used as conjugated antibodies; goat anti-human IgG (MABTECH, Nacka, Sweden), mouse monoclonal anti-human IgG2, IgG3 (Phamingen, USA), rabbit anti-human IgE (Vector Laboratories, USA).

Genotyping

DNA was extracted from packed red blood cells by standard phenol-chloroform extraction (Alloueche 2000). DNA was amplified by PCR and IL-4 –590 C/T alleles were detected by restriction fragment length polymorphism analysis as previously described (Gyan BA 2004). PCR reactions were carried out in 20 µl containing 1 µl DNA, 25 pmol each primer, 80 µmol each dNTP, 1.5 mM $MgCl_2$, and 0.5 U Taq polymerase (ABgene®, Biotechnologies Ltd, UK). The following primer sets were used, forward 5'AAA TAA AAA TAA AAA TGA GC 3' and reverse 5'CTG GGG AAA GAT AGA GTA ACT 3'. The amplification condition consisted of an initial 5-min denaturation at 95 °C, followed by 35 cycles of 95 °C for 30sec, 60 °C for 30sec, and 72 °C for 30sec, and finally a 5-min extension at 72 °C. The PCR products were digested by the restriction enzymes *BsmI*I (New England Biolabs Inc, USA) and visualized by UV light on ethidium bromide stained agarose gels.

Detection of specific IgG, IgG subclasses and IgE levels

Anti-*P.falciparum* IgG, IgG2, IgG3 and IgE antibodies levels were determined by enzyme-linked immunosorbent assay as previously described (Perlmann H 1994). Briefly, ELISA plates (Costar EIA, Cambrided, MA, USA) were coated with 50ul/well of a crude percoll-enriched lysate of parasite infected erythrocytes antigen (10 µg/ml) and blocked with 0.5 % w/v BSA in coating buffer for 3 hrs at 37°C. Samples were then added at different dilutions 1:1,000 for the determination of anti-*P. falciparum* IgG, 1:100 for IgG2, 1:1600 for IgG3 and 1:100 for IgE. Samples were determined in duplicates. The sera were allowed to react for 1 hr at room temperature for all determinations, with the exception of anti-*P. falciparum* IgE, which were incubated overnight. Bound IgG was then detected by adding goat-anti human IgG conjugated to alkaline phosphatase (ALP) (Mabtech, Nacka, Sweden). Anti-*P. falciparum* IgG2, IgG3, or IgE were detected with biotinylated (mouse monoclonal

anti-human IgG2 1: 3,000, IgG3 1 : 1,000 Pharmingen, USA, rabbit anti-human IgE 1: 8,000 Vector Laboratories, USA), followed by ALP-conjugated streptavidin (diluted 1: 2,000) (Mabtech, Nacka, Sweden). The optical density values were read in a Vmax Microplate Reader (Molecular Devices Corporation, Sunnyvale, CA) at 405 nm wavelength. The concentrations of anti-*P. falciparum* IgG, IgG2, IgG3 and IgE antibodies levels were calculated from standard curves obtained by incubating the coated plates with serial dilutions of either affinity-purified human serum IgE (National Institute for Biological Standards and Control, Hertfordshire, United Kingdom) or highly purified IgG (Jackson Immuno Research Laboratories, West Grove, PA) or purify myeloma IgG2, IgG3 isotype (serotec, Oxford, UK). IgG1 concentrations were determined by subtracting the levels of IgG2 and IgG3 from total IgG.

Statistical analysis

The data were analyzed using StatView computer software. Antibody levels are presented as geometric mean ± SE. The Mann-Whitney U test and the Kruskal-Wallis test were used to test the significant associations between anti – *P.falciparum* IgG2 and IgE plasma levels and IL4 genotype The Mann-Whitney U test was used to compare between 2 groups and the Kruskal-Wallis test compare categorial data. The relationship between IgG subclasses and IgE was examined using linear regression analysis. A p – value less than 0.05 was judged statistically significant.

RESULTS

IL4 –590 genotype and allele frequency in malaria patients

The genotype and allele frequencies of the IL4-590 polymorphism in complicated and uncomplicated Thai malaria patients were analyzed and illustrated in Table 1. The frequency of IL4 –590 T allele in the patients with complicated malaria did not differ from those of uncomplicated malaria (0.72 in both groups). Similar to the genotype distribution, the percentage of IL4 –590 genotypes in both groups was similar. The genotype distributions of IL4 -590 in complicated and uncomplicated malaria groups were consistent with Hardy-Weinberg equilibrium.

Anti- *P.falciparum* IgG subclasses and IgE

The geometric mean of anti-*P.falciparum* IgG subclasses and IgE antibodies on admission Day 0, Day 7, Day 28 in the patients with complicated and uncomplicated malaria were compared and shown in Figure. 1. Anti-*P.falciparum* IgG1 and IgG3 levels were significantly higher in the uncomplicated malaria patients as when compared to the complicated malaria patients in all time points tested i.e. Day 0, 7, and 28. No significant differences were seen in the levels of anti-*P.falciparum* specific IgG2 and IgE antibodies between complicated and uncomplicated malaria, except on Day 0, where the IgG2 levels were higher in the complicated malaria.

Association between concentration of anti-*P.falciparum* IgG subclasses and IL4 –590 gene polymorphism

The association between anti-*P.falciparum* IgG subclasses and IL-4 –590 genotype were analyzed. There was a significant higher IgG2 levels for carriers than non carriers of IL4 –590 C allele in the total study population (p = 0.038 for Day 0, p = 0.052 for Day 7, p = 0.052 for Day 28, respectively by Kruskal Wallis test). When subjects with complicated and uncomplicated malaria were analyzed separately. As can be seen in Figure 2. The complicated malaria patients carrying the IL4 –590 CC genotypes had significantly higher anti-*P.falciparum* IgG2 levels than those carrying the CT and TT genotypes. None of other IgG subclasses were significantly associated with the IL4 -590 genotypes neither in complicated nor in the uncomplicated malaria group.

Relationship between anti-*P.falciparum* IgG subclasses, IgE and IL4 –590 polymorphism

We investigate the relationship between anti-*P.falciparum* IgG subclasses and IgE levels. There was a significant correlation between anti-*P.falciparum* IgG2 and IgE levels in the complicated malaria group (R = 0.482, $p < 0.001$). Anti-*P.falciparum* IgG1 and IgG3 did not reveal any relation with IgE levels (data not shown). Further analysis reveal that the relationship between anti-*P.falciparum* IgG2 and IgE differ according to the IL4 –590 genotypes. The relationship between anti- *P.falciparum* IgG2 and IgE was stronger in the patients homozygous of C allele than those carrying C/T allele (IL4 –590 CC R = 0.902, p = 0.036, IL4 –590 CT R = 0.736, $p < 0.0001$ for

Day 0, IL4 –590 CC R = 0.856, $p = 0.029$; IL4 –590 CT R = 0.45, $p < 0.0025$ for Day 7). No significant correlation were observed between anti-*P.falciparum* IgG2 and IgE in the patients homozygous of the T allele (R = 0.021, $p = 0.882$ for Day 0, R = 0.064, $p = 0.662$ for Day 7) (Figure 3.).

DISCUSSION

This study did not detect any association between the IL4 –590 polymorphism and severity of malaria. However, we found that IL4 –590 C allele was associated with significantly elevated levels of anti – *P.falciparum* IgG2 antibodies. This association was observed in patients with complicated malaria, but not in those with uncomplicated malaria. Moreover, the elevated levels of anti – *P.falciparum* IgG2 were correlated with anti – *P.falciparum* IgE. None of other IgG subclasses were shown significantly associated with IL4 –590 polymorphism.

IL4, a Th2 cytokine, induces germline and mature transcripts corresponding to all seven human downstream Cγ1, Cγ2, Cγ3, Cγ4, Cα1, Cα2, and Cε gene and promotes Ig class switching to all Ig isotypes (Cerutii A 1998 2145, Litinskiy MB 2002 822). In human B cells IL4 is not specific for the ε or γ4 germline gene transcription and does not suppress γ1, γ2 or γ3. This is contrary to the reported situation in murine B cell where the Th1 cytokine IFN-γ antagonizes the IL4 response.(Billiau A 1996 61). Conversely, IFN-γ does not inhibit εGLT in single human B cells (Fear DJ 2004 4529). The IFN-γ, the prototypical Th1 cytokine, is essential for the IgG2 antibody response (Kavano Y 1994 4948, Kitani A 1993 3478). However, a defect in production of IFN-γ in selective IgG2 deficiency is still unclear. In patients with selective IgG2 deficiency, IFN-γ treatment did increase the production of IgG2 by 50 percent (Pan Q 2000 99). Recently, it has been shown that IFN-γ induces B cells to undergo isotype switching to IgG2, only in the concert with Th2 cytokine IL4 (Al-Darmaki S 2004 720) and this may at least partially attributed to the effects of IL4 as B cell growth factor (Cerutti A 1998 2145). Thus, both Th1 and Th2 cytokines are necessary for optimal IgG2 production.

The relationship between IL4 polymorphisms and anti - *P.falciparum* IgG2 plasma levels has not been studied thoroughly. In the present study, our data showed that a single nucleotide polymorphism at position IL4 –590 transition from C to T, related to the transcription start site, associated with elevated plasma anti-*P.falciparum* IgG2 levels. The IL4 –590 CC carrier showed significantly higher plasma anti-*P.falciparum* IgG2 levels than those with CT and TT carrier. The association was observed only in those with complicated malaria. This may partially explain the association between anti-*P.falciparum* IgG2 response and number of malaria attacks or disease (Chumpitazi BFF 1996 151, Ndungu FM 2002 77). The IgG2 antibody response tends to be elicited against carbohydrate antigens, and significantly stimulated by IFN-γ. Carbohydrate antigens, such as lipopolysaccharide (LPS) induce dendritic cells (DCs) to secrete IL12 which in turn and in turn activates lymplocytes to produce IFN-γ. Th1 cytokine IFN-γ synergises with IL4 to induce isotype switching to IgG2 (Al-Darmaki S 2004 720, Kepsenberg LM 2003 984). Thus, besides IL4 –590 C/T polymorphism other Th1 associated cytokine gene polymorphisms as IFN-γ, IL12, IL18 and their signaling genes may also involve in the regulation of IgG2 response and may contribute to regulate severity or protection of the disease.

In the present study, a relationship between anti-*P.falciparum* IgG2 and IgE among patients with complicated malaria was observed in IL4 –590 C carrier. The correlation between the IgG2 and IgE levels was stronger in the patients being C homozygous than those being C heterozygous, no significant correlation between anti-*P.falciparum* IgG2 and IgE levels in the patients homozygous for the T allele were seen. Thus our data suggest that the IL4 –590 C allele is involved in the regulation of anti-*P.falciparum* IgG2 and IgE antibodies production. In the present study, we demonstrate that IL4 –590 CC genotype was associated with plasma anti-malaria IgG2 levels in complicated malaria, but not anti-*P.falciparum* IgE levels In this regard, anti-*P.falciparum* IgE levels may be too low to reach statistical significance (Perlmann H 1994 284). However, the correlation between anti-*P.falciparum* IgG2 and IgE were observed. Thus, these may indicate the association between IL4 -590 C carrier and anti – malaria IgE antibody response.

Previous study has been revealed that the IL4 –590 T allele associated with total IgE antibody production (Verra F 2004 205, Gyan BA 2004 145). The association between total IgE and the IL4 –590 seem to vary depending on the genetic heterogeneity of the study populations. The association between IL4 –590 T and total serum IgE have found in American white (Rosenwasser LJ 1995 74), but not in Australian and British white or Japanese children (Walley AJ 1996 689, Noguchi E, 1998 449). IL4 –590 T allele in Thai (72%) is much higher than in Africa population (10-15%), which might explain why the association of IL4 –590 T with total IgE found in African individuals was not found in Thai individuals. Another finding has showed that IL4 –590 T allele associated with total anti – malaria IgG antibodies and more protected to malaria (Luoni G 2001 411). The total anti – malaria IgG represent of the IgG1 response, which is contradict to our finding. We demonstrated that IL4 – 590 C allele was associated with anti-*P.falciparum* IgG2 levels in patients with complicated malaria. These anti-*P.falciparum* IgG2 antibodies were observed to correlated with anti-*P.falciparum* IgE antibody production. The gene encoding IgG2 and IgE are located at the second block of Ig gene. Thus, it is likely that IgG2 and IgE class switching was induced by similar gene polymorphism, IL4-590 C allele. Anti-*P.falciparum* IgG2 and IgE antibodies may antagonize the protective cytophilic IgG1 and IgG3 antibodies and might thus reduce parasite clearance (Bouharoun-Tayoun H 1992 1473, Tebo AE 2001 98). In addition, IgE mediated cross linking of CD23 induced TNF-α, which could be an important protective mechanism in malaria infection. As the same time, the over production may contribute to the pathogenesis of the disease (Perlmann P 1997 116). The regulation of Ig classes and subclasses production via IL4 polymorphism is particularly interested and required further investigation.

Our results suggested that the –590 polymorphism of IL4 gene participated in the regulation of the IL4 response, which in turn may induce plasma anti – *P.falciparum* IgG2 and IgE levels found in complicated malaria. Anti – *P.falciparum* IgG2 and IgE levels were increased in complicated malaria patients with IL4 –590 C allele. This polymorphic was not associated with anti – *P.falciparum* IgG2 and IgE levels in patients with uncomplicated malaria. A cascade of cytokines were produced in acute malaria infection, clinical malaria manifestations may reflect the unbalance of the Th1

and Th2 immune response. Thus, other key genes and the combination of base change of several sites in the promoter, may also participate in the regulation and contribute to development of complicated or uncomplicated malaria. The association between IL4 –590 C allele and plasma anti – *P.falciparum* IgG2 and IgE levels may represent genetic markers predisposition of disease severity. Furthermore, the understanding of IL4 polymorphism and the regulation of antibody response may help in deciding which patients are more likely to benefit from a vaccine or life threatening.

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SIGNIFICANCE OF THE RESEARCH

1.The present study will be published in the international journal as the following "Anti - *P.falciparum* IgG2 and IgE antibodies and IL4 polymorphism related to severe malaria in Thai population" *(submitted to Am J Trop Med Hygiene)*

2. Our results may valuable in prescribing appropriate anti-malarial drugs or deciding which patients are most likely to benefit from a vaccine or life threatening. Since the single nucleotide cytokine gene polymorphisms may represent as genetic markers of severity of the disease, malaria.

FURTHER STUDY

Severe malaria is the outcome of a complex cascade of cytokines production. The relationship between cytokine expression and the single polymorphic site is not as simple as might be expected. Rather than a single polymorphic site, the combination of base changes at several sites on the promoter, i.e. the haplotype need to be further investigated for their effects in cytokines expression. These markers may contribute to the outcome of severe or mild malaria.

ภาคผนวก

The manuscript is being submitted for publication in international journal and as the report above.

Table 1. Genotype and alleles frequencies of IL4 -590 C/T polymorphism in complicated and uncomplicated Thai malaria patients

IL4 -590 C/T	Complicated (110)	Uncomplicated (166)
GENOTYPE		
TT	55 (0.500)	86 (0.518)
TC	49 (0.445)	69 (0.415)
CC	6 (0.054)	11 (0.066)
ALLELE		
T	61 (0.723)	91 (0.726)
C	159 (0.277)	241 (0.274)

Frequencies are in the parenthesis.

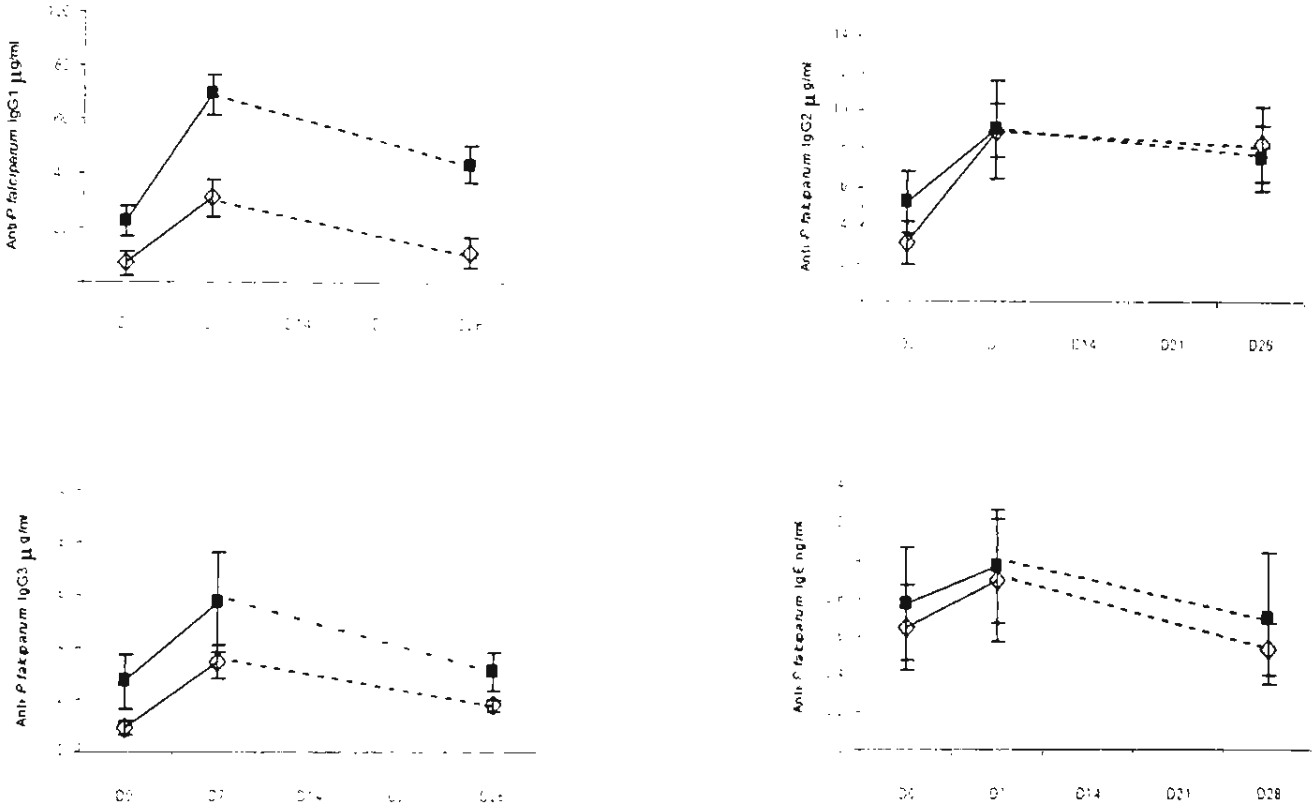

Figure 1. Geometric means of IgG1, IgG2, IgG3, and IgE antibodies in sera from patients with severe (empty squares) or uncomplicated (black square) *Plasmodium falciparum* malaria. D0 = day of admission.

Figure 2 Anti-*P.falciparum*IgG2 and IL-4 590 relationship in complicated malaria

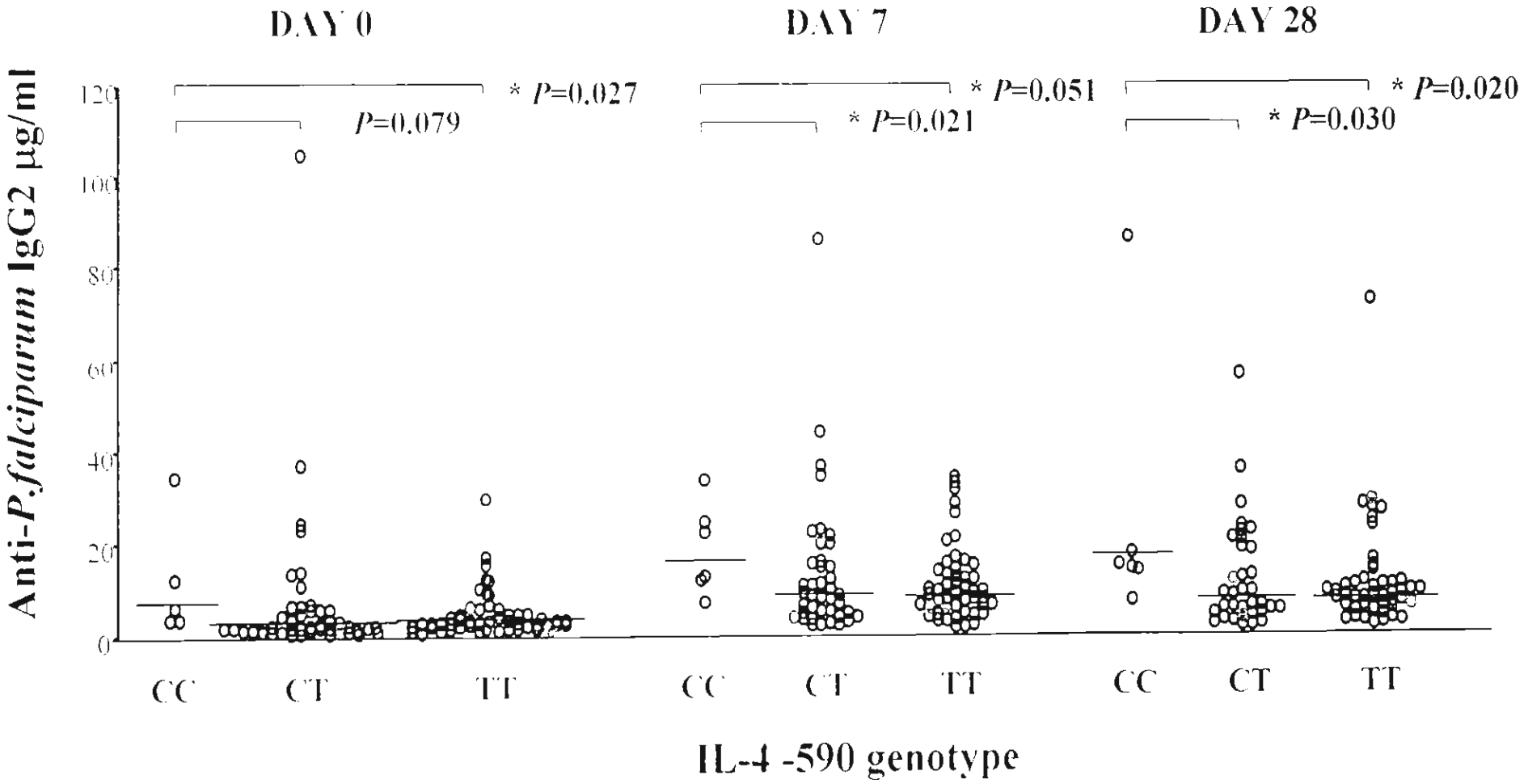

Figure.3 Relationship between anti - *P.falciparum* IgG2 and IgE and the IL-4-590 genotype in complicated malaria