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Association between Single-Nucleotide Polymorphism (rs2072493) and Serum Level of TLR-5 and Interleukin-6 and Interleukin-12 Response to *Toxoplasma gondii* in Women with Miscarriage and Pregnant Women

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Abstract

Background *Toxoplasma gondii* (*T. gondii*) is well-known to cause congenital diseases, or ocular diseases, and miscarriage in pregnancy. Earlier infections are threatening in pregnant women. The activity of Toll-like receptors (TLR) in defense against *T. gondii* infections was observed such as TLR-5 molecule, and some interleukins (like IL-6, and IL-12) that may lead to abortion.

Objective To study association between TLR-5 gene polymorphisms (rs2072493) with the susceptibility to toxoplasmosis in a sample of women with miscarriage and to study the association of TLR-5 polymorphisms (rs2072493) with the serum level of TLR-5, IL-6 and IL-12.

Methods This is a case-control study was conducted on 200 women, of which, 50 pregnant women seropositive (IgG, IgM) for *T. gondii* (Group1), 50 pregnant women seronegative (IgG, IgM) for *T. gondii* (Group2) as a control group, 50 women with miscarriage seropositive (IgG, IgM) for *T. gondii* (Group3), and 50 women with miscarriage seronegative (IgG, IgM) for *T. gondii* (Group4) as a control group. They were recruited from Balad General Hospital, Salah al-Din and private laboratory in the period from January 2021 to December 2021. The serum level of TLR-5, IL-6 and IL-12 were measured. The selected single-nucleotide polymorphism (SNPs) (rs2072493) in TLR-5 were detected by using real-time polymerase chain reaction with specific primers.

Results The frequency of the heterozygous genotype (TC) for SNPs TLR-5 gene (rs2072493) was significantly higher in Group1 than Group2 (P=0.004). At allelic level, the frequency of mutant allele (allele C) was significantly higher in Group1 than in Group2 (p=0.009), and in Group3 than in Group4 (P=0.025). There was significant increase in serum level of TLR-5 in Group1 and in Group3. There was significant increase in serum level of IL-6 and IL-12 in Group1.

Conclusion There is significant association of toxoplasmosis with mutant allele (allele C) of the SNP rs2072493, which may be considered as a risk factor for toxoplasmosis and stimulate miscarriage in pregnant women. TLR-5 level is high in both groups.

Keywords TLR-5, polymorphism, Toxoplasma gondii, IL-6, IL-12

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List of abbreviations: ELISA = Enzyme linked immunosorbent assay, IL = Interleukin, IFN = Interferon, IgM = Immunoglobulin, IgG = Immunoglobulin γ, NF-κB = Nuclear factor kappa, SNPs = Single-nucleotide polymorphisms, Th2 = T helper, TLRs = Toll-like receptors

Introduction

Toxoplasmosis infection is rarely symptomatic in immune - competent individuals, but in immunocompromised

host may result in a severe disease or even fatal damage [1]. Toxoplasmosis is caused by *Toxoplasma gondii* (*T. gondii*), which is an obligate intracellular parasite, and considered the most public global parasite [2]. It is one of the most common infections during pregnancy that are passed from mother to child. As well as is the main factor in perinatal morbidity and mortality [3].

Profilin, a pathogen-associated molecular pattern (PAMP) produced by *T. gondii*, is recognized by receptors on dendritic cells and macrophages, activating the cells and causing the release of proinflammatory cytokines like interleukin (IL)-6 and IL-12 [2].

It has been demonstrated that activating Toll-like receptor (TLR)-11 by profilin causes powerful production of protein from mouse dendritic cells, but the human TLR-11 gene contains many stop codons, which lead to transcription of it does not produce a functional protein [4].

The TLR phylogenetic tree has an ancient cluster that includes both human TLR-5 and mouse TLR-11; as a result, human TLR-5 may have preserved mouse TLR-11's biological function and mediated *T. gondii* profilin identification [5,6]. The ectodomain of human TLR-5 contains shared binding sites for flagellin and profiling [6].

TLR-5 is encoded by a gene that has six exons and is located on the long arm of human chromosome 1 (hCh1q). There are now nine reported possible polymorphisms in the gene's promoter and coding regions [7], the functional TLR gene polymorphisms (rs2072493) are within exon region [8].

This research aimed to:

1. Study the association between TLR-5 gene polymorphisms and the susceptibility to toxoplasmosis in a sample of women with miscarriage.
2. Study the association of TLR-5 polymorphisms with the serum level of TLR-5 and cytokines (IL-6 and IL-12).

Methods

The study was approved by the Institutional Review Board (IRB) in the College of Medicine, Al-Nahrain University. The present Case-control study was conducted on 200 women, of which 50 pregnant women seropositive (IgG, IgM) for *T. gondii* (Group1), 50 pregnant women seronegative (IgG, IgM) for *T. gondii* (Group2) as a study group, 50 women with miscarriage seropositive (IgG, IgM) for *T. gondii* (Group3), and 50 women with miscarriage seronegative (IgG, IgM) for *T. gondii* (Group4) as a study group; which were collected in the period from January 2021 to December 2021.

All women participated in the study were recruited from Obstetrics and Gynecology Department of Balad General Hospital in Balad city, Salah al-Din province/Iraq, and all women participating in the study examined by a consultant specialist of gynecologist and obstetrician.

Five ml of whole venous blood was taken from each patient, and patients' information was taken from the data recorded. For the purpose of extracting DNA, two ml of ethylene diamine tetraacetic acid (EDTA) was collected, and both the EDTA tube and the extracted DNA were preserved at -20 °C until use. The remaining three ml of blood were collected in a plain gel tube for serum separation and kept at -20 °C until needed. All women participating in the study were investigated for the presence of anti-*T. gondii* antibodies by rapid chromatographic immune technique, and confirmed by enzyme linked immunosorbent assay (ELISA) technique. To confirm the diagnosis of toxoplasmosis, ELISA was used to detect IgM and IgG levels in all samples, whether they were positive or negative for anti-*T. gondii* Abs. The serum level of TLR-5 and cytokines (IL-6, IL-12) were measured by ELISA technique. Real-time polymerase chain reaction (RT-PCR) was used with particular primers to detect the selected Single-nucleotide polymorphisms (SNPs) (rs2072493) in TLR-5.

Statistical analysis

The program (Graph Pad Prism version 7) was utilized, and the one-way ANOVA (by Tukey's multiple comparisons test) was used to compare the observed parameters and SNP numbers between subdivided groups. Mean Standard Error was used to represent the results. Using the Chi-square test, nominal variables were given as frequency and percentage (%) and compared between study groups.

The MedCalc tool was used to calculate the odds ratios (OR), 95 % of confidence intervals (CI), and p values used to express the results of nominal regression. Significance of differences was determined at ($p < 0.05$). To determine the correlation between markers, correlation coefficients were computed. Mega Stat (Version v 10.12) for Excel 2010 was used to calculate the descriptive statistics and correlation coefficients (9).

Results

Age groups and study groups

The mean age of study groups were 30.36± 0.43 years, the minimum age was 18 years and the maximum age was 43 years (Table 1).

Table 1. Age groups distribution among study groups

Groups	Age groups (years) <20 N (%)	20-30 N (%)	31-40 N (%)	>40 N (%)	Total N (%)
Group1	2 (4)	25 (50)	21 (42)	2 (4)	50 (100)
Group2	3 (6)	26 (52)	21 (42)	0 (0.0)	50 (100)
Group3	0 (0.0)	24 (48)	23 (46)	3 (6)	50 (100)
Group4	0 (0.0)	21 (42)	27 (54)	2 (4)	50 (100)

Anti-*T. gondii* antibodies

According to cassette screening test, 32 (64%) women with miscarriage were positive for both anti-*T. gondii* IgM and IgG antibodies, 5 (10%) of them were positive for anti-*T. gondii* IgM antibodies only, and 13(26%) of them were positive for anti-*T. gondii* IgG antibodies only, while 21 (42%) pregnant women were positive for both anti-*T. gondii* IgM and IgG antibodies, 7 (14%) of them were positive for anti-*T. gondii* IgM antibodies only, and 22 of them (44%) were positive for anti-*T. gondii* IgG antibodies only.

Positive for anti-*T. gondii* IgM antibodies acute cases were excluded, because its percentage is low and no statistically significant and only chronic cases were taken, therefore, the fifty women with miscarriage with (IgM, IgG) seropositive for *T. gondii* and fifty pregnant women with (IgM, IgG) seropositive for *T. gondii* by cassette screening test were positive for anti-*T. gondii* (IgM, IgG) antibodies (100%) by ELISA, while the controls groups were negative result (0%) have for that antibodies as shown in table (2).

TLR-5 gene polymorphism rs2072493

Genetic polymorphism of TLR-5 gene (rs2072493) was observed with three genotypes (TT, TC and CC) (Figure 1). Compared to Group2, which had a homozygous genotype (TT) frequency of 48(96%), Group1 had a homozygous genotype (TT) with a frequency of 30(60%), revealed a high significant difference (P=0.0004) (OR=0.06, CI=0.01 to 0.28), and the frequency of the heterozygous genotype (TC) was higher in group1 20 (40%) than Group2 2 (4%) with high significant difference (P = 0.0004) (OR = 16, CI = 3.48 to 73.4), but homozygous genotype CC was not recorded any frequency 0(0%) in both groups (Table 3).

Table 2. Anti-*T. gondii* antibodies

Groups	Cassette test IgM, IgG N (%)	Cassette test IgM N (%)	Cassette test IgG N (%)	ELISA IgM, IgG N (%)
Group1	21 (42.0)	7 (14.0)	22 (44.0)	50 (100)
Group2	0 (0.0)	0 (0.0)	0 (00.)	0 (0.0)
Group3	32 (64.0)	5 (10.0)	13 (26.0)	50 (100)
Group4	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Total		100		100

P value = 0.6441

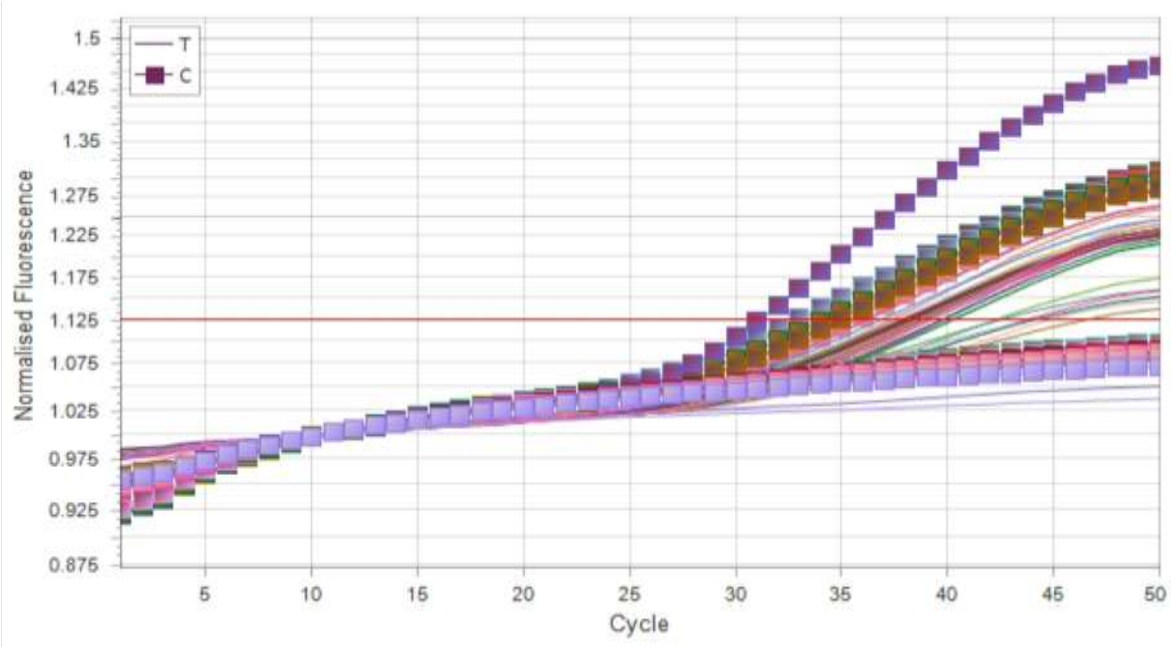

Figure 1. RT-PCR Result for Genetic Polymorphism of TLR-5 gene (rs2072493)

Table 3. Genotypes and alleles of TLR-5 gene polymorphism rs2072493 in group1 and group2

	rs2072493	Group1 (n =50)	Group2 (n =50)	OR (95 % CI)	P value
Genotypes	TT	30 (60%)	48 (96%)	0.06 (0.01 to 0.28)	0.0004
	TC	20 (40%)	2 (4%)	16.00 (3.48 to 73.41)	
	CC	0 (0.0%)	0 (0.0%)	--	
Alleles	T	80 (80%)	98 (98%)	0.08 (0.01 to 0.35)	0.0009
	C	20 (20%)	2 (2%)	12.25 (2.77 to 53.99)	

At allelic level, the frequency of normal allele (allele T) was in group1 and group2 (80% versus 98%) with high significant difference (P = 0.0009) also (OR = 0.08, CI = 0.01 to 0.35), while in mutant allele (allele C) was in group1 and group2 (20% versus 2%) with high significant difference (p=0.0009) also (OR = 12.25, CI = 0. 2.77 to 53.99), as shown in table (3).

Also, the frequency of the homozygous genotype (TT) frequency 41 (82%) was not significant P = 0.07 (OR = 0.29, CI = 0.07 to 1.14) in group3 compared to Group4 47 (94%), and the heterozygous genotype (TC) was higher in group3 6 (12%) than group4 3(6%) with no significant difference P = 0.30 (OR = 2.13, CI = 0.50 to 9.06), while the homozygous genotype CC frequency was no significant difference P = 0.18 (OR = 7.44, CI = 0.37 to 147.93) in Group3 3 (6%) compared to Group4 0 (0%), the frequency of normal allele (allele T) at allelic level was in group3 and group4 (88% versus 97%) with significant difference P = 0.025 also (OR = 0.22, CI = 0.06 to 0.83), while in mutant allele (allele C) was in Group3 and Group4 (12% versus 3%) with significant difference P = 0.025 also (OR = 4.40, CI = 1.20 to 16.14), as shown in table (4).

Table 4. Genotypes and alleles of TLR-5 Gene Polymorphism rs2072493 in group3 and group4

	rs2072493	Group3 (n =50)	Group4 (n =50)	OR (95 % CI)	*P-value*
Genotypes	TT	41 (82%)	47 (94%)	0.29 (0.07 to 1.14)	0.07
	TC	6 (12%)	3 (6%)	2.13 (0.50 to 9.06)	0.30
	CC	3 (6%)	0 (0.0%)	7.44 (0.37 to 147.93)	0.18
Alleles	T	88(88%)	97 (97%)	0.22 (0.06 to 0.83)	0.025
	C	12 (12%)	3 (3%)	4.40 (1.20 to 16.14)	

The serum soluble level of TLR-5, IL-6 and IL-12

Table 5 shows the results of TLR-5, IL-6 and Il-12, they were significantly higher in group1 (mean = 48.43, 151.1 and 27.71) than group2 (mean = 14.72, 151.1 and 27.71) respectively, also, they were significantly higher in group3 (mean = 28.30, 138 and 30.91) than group4 (mean =10.92, 91.89 and 24.63) respectively.

Table 5. The serum soluble level of TLR-5, IL-6 and Il-12

Groups	TLR-5 (ng/l) Mean±SE	IL-6 (ng/l) Mean±SE	IL-12 (ng/l) Mean±SE
Group1	48.43±3.7	151.1±17.42	27.71±2.22
Group2	14.72±2.2	83.45±7.5	15.77±2.28
Group3	28.30±1.93	138±13.36	30.91±2.62
Group4	10.92±1.86	91.89±9.74	24.63±3.75
P value	<0.0001	0.0002	0.0013

P value: one-way ANOVA

Correlations among TLR-5, IL-6 and IL-12 and rs2072493 in group1

As shown in table (6), the results of the present study revealed a non-significant negative correlation (P >0.05) among TLR-5, IL-6 and rs2072493, and a non-significant positive correlation (P>0.05) between IL-12 and rs2072493; also, a non-significant positive correlation (P>0.05) among TLR-5, IL-6 and IL-12, while showed a significant positive correlation (P<0.05) between IL-6 and IL-12.

Table 6. Correlations among TLR-5, IL-6 and IL-12 and rs2072493 in group1

		rs2072493	TLR-5	IL-12	IL-6
rs2072493	r	1.000	-0.255	0.076	-0.025
	P value	.	0.074	0.598	0.861
TLR-5	r		1.000	0.070	0.142
	P value		.	0.627	0.325
IL-12	r			1.000	0.831**
	P value			.	0.000

** = Spearman's rho Correlation is significant at the 0.05 level (2-tailed)

Correlations among TLR-5, IL-6 and IL-12 and rs2072493 in group2

As shown in table (7) the results of the present study revealed a non-significant positive correlation and difference (P>0.05) among TLR-5, IL-6, IL-12 and rs2072493, and a non-significant negative correlation (P>0.05) between TLR-5 and IL-12, also, a significant positive correlation (P<0.05) between TLR-5 and IL-6, moreover, a non-significant positive correlation and no significant difference (P>0.05) between IL-6 and IL-12.

Correlations among TLR-5, IL-6 and IL-12 and rs2072493 in Group3

As shown in table (8), the results of the present study revealed a non-significant negative correlation (P>0.05) among TLR-5, IL-6, IL-12 and rs2072493, and a significant positive (P<0.05) among TLR-5, IL-6 and IL-12; also, a significant positive correlation (P<0.05) between IL-6 and IL-12.

Table 7. Correlations among TLR-5, IL-6 and IL-12 and rs2072493 in group2

		rs2072493	TLR-5	IL-12	IL-6
rs2072493	r	1.000	0.202	0.014	0.057
	P value	.	0.160	0.922	0.696
TLR-5	r		1.000	-0.032	0.745**
	P value		.	0.828	0.001
IL-12	r			1.000	0.120
	P value			.	0.405

** = Spearman's rho Correlation is significant at the 0.05 level (2-tailed)

Table 8. Correlations among TLR-5, IL-6 and IL-12 and rs2072493 in group3

		rs2072493	TLR-5	IL-12	IL-6
rs2072493	r	1.000	-0.240	-0.179	-0.247
	P value		0.094	0.214	0.084
TLR-5	r		1.000	0.373**	0.554**
	P value		.	0.008	0.000
IL-12	r			1.000	0.741**
	P value			.	0.000

** = Spearman's rho Correlation is significant at the 0.05 level (2-tailed)

Correlations among TLR-5, IL-6 and IL-12 and rs2072493 in group4

As shown in table (9), the results of the present study revealed a non-significant positive correlation (P>0.05) among TLR-5, IL-6 and rs2072493, and a non-significant negative correlation (P>0.05) between IL-12 and rs2072493; and a significant positive correlation (P<0.05) between TLR-5 and IL-6, also, a non-significant negative correlation (P>0.05) between TLR-5 and IL-12; in addition, a non-significant positive correlation (P>0.05) between IL-6 and IL-12.

Table 9. Correlations among TLR-5, IL-6 and IL-12 and rs2072493 in group4

		rs2072493	TLR-5	IL-12	IL-6
rs2072493	r	1.000	0.096	-0.044	0.108
	P value	.	0.506	0.763	0.455
TLR-5	r		1.000	-0.085	0.722**
	P value		.	0.559	0.001
IL-12	r			1.000	0.013
	P value			.	0.927

** = Spearman's rho Correlation is significant at the 0.05 level (2-tailed)

Discussion

Maternal acute toxoplasmosis or congenital toxoplasmosis during pregnancy are two of the main factors that raise the risk of abortion. Toxoplasmosis has a significant frequency worldwide, according to serological evidence [10-12]. Innate immunity has a major role in toxoplasmosis and recurrent miscarriages, including TLR-5 and some cytokines, since both mouse and human TLR-5 appear to be the earliest evolutionary relatives of mouse TLR-11, human TLR-5 may have preserved mouse TLR-11's biological function and mediated the identification of *T. gondii* profilin. The ectodomain of human TLR-5 contains shared binding sites for flagellin and profilin [5].

TLR-5 might play an important role in the pathogenesis of unexplained recurrent spontaneous abortion since TLR-5 signaling could result in inflammatory cytokine production (such as IL-6 and IL-12) [13], especially when there is no reason for the miscarriages, so *T. gondii* infection could be considered a potential risk factor for abortion. Host genetics play a key role in determining disease susceptibility, clinical symptoms, treatment response, and disease outcome in addition to the pathogen's virulence characteristics.

Age groups and study groups

The current study showed the distribution of toxoplasmosis among age groups was converged. These findings agreed with Ahmed et al. in 2019, who reported that the distribution among these ages was converged, may be because the women participating in this study were in the reproductive stage [14].

Anti-*T. gondii* antibodies

The current study revealed that the anti-*T. gondii* IgM positivity was too low among study groups, 7(14%) in group1, and 5 (10%) in group3, these results are disagreed with Turkey et al. in 2019, who proved that 33 (66%) of aborted women were positive for IgM antibodies [15], this disagreement might because that the current study has been conducted on women with chronic toxoplasmosis only.

TLR-5 gene polymorphism

Throughout the human genome, a huge number of single nucleotide polymorphisms (SNPs) have been discovered. SNPs are becoming more important and useful in the search for the causes of human diseases and features, as well as in drug development and the research of human treatment response and may affect the innate immune response, by changing the amplitude and quality of intracellular signaling cascades, which has consequences for infection susceptibility, and disease results. This is backed up by a growing body of evidence [16].

Furthermore, there was a significant association between the heterozygous genotype (TC) of TLR-5 gene polymorphism rs2072493 and susceptibility to toxoplasmosis in group1. This suggests that carrier of (TC) genotype of this polymorphism are higher risk of having the disease, compared with (TT) genotype carrier, causes according to odd ratio by 16 under 95% CI (3.48 to 73.4). While it showed no significant difference between the heterozygous genotype (TC) of TLR-5 gene polymorphism rs2072493 and susceptibility to *T. gondii* in Group 2.

There are very a few studies that have researched into this topic (TLR-5 gene polymorphism with toxoplasmosis) globally. The detected allele frequency for (rs2072493) polymorphism in the current study were 40% in group1, these results disagreed with some researches, who reported that allele frequencies of 15% in Caucasians [17], Chinese were 26% [18], and north Indians were 12 % [19], may be because these researches used a population large sample, as well the genetic heterogeneity among different ethnicities, variety can be attributed to the differences in the minor allele incidences, and SNP rs2072493 was associated with various infectious such as colorectal cancer, graves' disease, and chronic hepatitis B virus (HBV) infection [20- 22].

The serum soluble level of TLR-5

In the present study, the serum soluble level of TLR-5 was significantly increased in group1 ($P< 0.05$), this results were agreed with Dabagh-Gorjani et al. in 2014, who indicated that the expressions of TLR-5 was significantly increased in both maternal part (the maternal and fetal parts of the placenta), although it used the expression for TLR-5, the results were agree, TLR-5 ligation activates the production of proinflammatory cytokines via the nuclear factor kappa (NF-κB) pathway, regardless of the trigger factors or ligands, and chronic inflammation is thought to be a primary contributor to the progression of abortion [23].

According to Salazar Gonzalez et al. in 2014, this result is in accordance with the theory that human TLR-5 is involved in innate recognition and initiation of cytokine production by *T. gondii* – derived profiling [6].

The serum level of IL-6

In the current study, IL-6 serum level showed a significant increase ($P<0.05$) more in the serum of group1, this finding was agreed with Zhang et al. in 2017, and Tyagi and Alharthi in 2020, who indicated that plasma concentrations of IL-6, IL-10, and IL-18 are higher in women with successful pregnancies than in women with recurrent pregnancy loss, and IL-6 was decreased in pregnant women with a history of

recurrent spontaneous miscarriage patients [24,25].

IL-6 plays a role in trophoblast proliferation, differentiation, and invasion, as well as follicle development and embryonic implantation, the IL-6 protein is also involved in the first spiral artery remodeling process, which necessitates the production of vascular smooth muscle cells and morphological changes. IL-6 levels that are lower inhibit trophoblast invasion and spiral artery remodeling [26]. Therefore, in this regard, the current result is consistent with numerous earlier investigations [27,28].

The general rule is that T helper2 (Th2) cytokines, such as IL-6, are thought to encourage a normal pregnancy, IL-6 functions as a messenger for notifying the body to the occurrence of an unexpected event. In an infected lesion, IL-6 is produced and transmits a warning signal throughout the body. Increased or decreased levels of this cytokine in serum or gestational tissues appear to have negative consequences for pregnancy. Excessive IL-6 may limit the development of cluster of differentiation (CD4+) T regulator cells, which are essential for pregnancy tolerance, according to one theory [29].

The serum level of IL-12

In the current study, the serum level of IL-12 showed a significant increase ($P<0.05$) more in the serum of group1, this result was disagreed with Tyagi and Alharthi in 2020, who reported that Th1 activity (including IL-12) was higher in pregnant women with a history of recurrent spontaneous abortion irrespective of whether continuing their pregnancy or aborting in contrast to healthy pregnant, maybe this disagree because that might be a persistent imbalance of Th1/Th2 in pregnant women [25].

The generation of interferon-gamma (IFN-γ) by interleukin-12 (IL-12) causes Th1 cells to differentiate. Researchers have discovered that Th1-type immunity may be harmful during pregnancy and that an increased Th1-type cytokine response may result in pregnancy loss because Th1-dependent processes are likely involved in allograft rejection [24], also, according to a study by Rezende-Oliveira et al. in 2012, who suggested that immunomodulation, which was observed during pregnancy, was involved in *T. gondii* evading the immune response [30].

IL-12 level in the blood were shown to be higher in failing pregnancies than in normal pregnancies. Important regulatory role was played by cytokines during pregnancy; an excessive decrease or increase of these cytokines may result in spontaneous abortion, implantation failure, or preeclampsia in patients with a history of miscarriage [31].

Therefore, in this regard, the current result is consistent with numerous earlier investigations [25,30,31]. This concordance, however, does not fit the IL-12 results in group2, may be because different in study group.

According to our knowledge, there is not any previous studies included study the correlations among TLR-5, IL-6 and IL-12 and SNPs rs2072493 in pregnant women. To the best of our knowledge, this study is the first to suggest.

In conclusions, firstly, there is a significant correlation between the heterozygous genotype (TC) of TLR-5 gene polymorphism rs2072493 and susceptibility to toxoplasmosis in pregnant women seropositive group, but there is no significant difference in women with miscarriage seropositive. Secondly, there is a significant increase serum soluble level of TLR-5 in both group women with miscarriage seropositive and of pregnant women seropositive. Thirdly, there are a significant increase serum level of IL-6 and IL-12 in pregnant women seropositive, but there are no significant in women with miscarriage seropositive. Lastly, both women with miscarriage seropositive and pregnant women seropositive with rs2072493 in the current study shows a negative correlation between TLR-5 and IL-6, but IL-12 was negatively correlation in women with miscarriage seropositive, and positive correlation in pregnant women seropositive.

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Author contribution
Dr. Al-Baldawy: did the laboratory work, wrote the article, and statistical analysis. Dr. Al-Marsomy: designed, supervised and co-wrote this article. Dr. Khaleel: diagnosed and follow up patients, and cooperated in samples collection.

Conflict of interest
The authors declare that they have no conflict of interest.

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