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Immunogenetic Interplay of *IL-6* and *IL-6R* Genes Variants and Circulating *IL-6* Levels in *Toxoplasma gondii*-infected women with Recurrent Pregnancy Loss

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ABSTRACT

Background: Recurrent pregnancy loss (RPL) is a global health challenge in women. *Toxoplasma gondii (T. gondii)* infection and interleukin-6 (*IL-6*) bioavailability contribute to RPL.

Objective: This study investigated *IL-6* and *IL-6* receptor (*IL-6R*) genes polymorphisms and blood *IL-6* levels in *T. gondii*-infected women with RPL.

Methods: The study comprised 163 women, including 83 infected (Group-A: subgroups A1, A2) and 80 healthy (Group-B). Demographic data, blood, and placental tissue samples were collected. The blood samples were screened for *IL-6* levels through ELISA. DNA was extracted, and *T. gondii*-DNA was identified using PCR. *IL-6* and *IL-6R* genes were amplified by PCR and sequenced using Sanger method.

Results: The *IL-6* gene -174G/C (p=0.005) and -597G/A (p=0.0012) polymorphisms were significantly, while -572G/C polymorphism was insignificantly (p=0.143) associated with infection and RPL. The mean blood *IL-6* level was significantly variable in all the groups (p<0.001). The *IL-6* gene -174GG and -597GG genotypes and the *IL-6R* gene AA genotype were common in Group-A2 (47.5%, 37.5%, 50%) and Group-B (56.2%, 60%, 68.8%) respectively, and also significantly associated with high blood *IL-6* levels. The -572GG genotype was common and insignificantly linked with elevated blood *IL-6* levels in all the groups (p=0.143). The *IL-6* gene -174CC and -597AA genotypes and *IL-6R* gene CC genotype were highly prevalent in Group-A1 (48.8%, 41.9%, 51.1%, respectively) and associated with low blood *IL-6* levels.

Conclusion: The *IL-6* gene -174G/C, -597G/A, and *IL-6R* gene A/C polymorphisms play significant roles in RPL in women infected with *T. gondii,* and change *IL-6* bioavailability.

Keywords: *IL-6*; *IL-6R*; SNP; *T. gondii;* RPL

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INTRODUCTION

Recurrent pregnancy loss (RPL) is a complex reproductive disorder characterized by two or more failed pregnancies before the 20th week of gestation according to American Society for Reproductive Medicine (ASRM). Globally, an estimated 23 million cases of pregnancy losses occur annually, with RPL affecting approximately 2 to 5% of women of reproductive-age (1). Multiple factors contribute to the development of RPL, including infectious agents, immune dysfunction and genetic abnormalities (2). *Toxoplasma gondii (T. gondii)* is an obligate intracellular protozoan parasite that causes a zoonotic disease, Toxoplasmosis, which can lead to serious complications during pregnancy. It is estimated that approximately one-third of the global human population has been exposed to or infected with T. gondii (3). The parasite shows high prevalence in Pakistan, with reports spanning multiple geographical regions of the country (4-12). Most immuneocompetent individuals remain asymptomatic following infection; however, severe or symptomatic disease typically occurs in immune-compromised individuals (13). The severity and risk of Toxoplasmosis are influenced by the maternal immune status, genetic background, and gestational age at the time of infection. The rate of maternal–fetal transmission increases with advancing gestation, whereas infection during early pregnancy is generally associated with more severe fetal complications including miscarriage and spontaneous abortion (14). *T. gondii* can be transmitted to humans through the consumption of contaminated food and undercooked meat containing cysts. It can also be transmitted vertically from an infected mother to the fetus, potentially leading to severe pregnancy-related complications (15). Toll-like receptors (TLRs), expressed on both immune and non-immune cells, play an important role in pathogen recognition and trigger celular activation and the cytokines production, including interleukin-6 (IL-6) (16).

IL-6 is a multifunctional acute-phase cytokine that plays a key role in infection control and the maintenance of pregnancy. The *IL-6* gene is located on chromosome 7 (7p15.3) and consists of five exons and four introns (17). *IL-6* is produced by various cell types, including dendritic cells, myocytes, leucocytes, adipocytes, osteoblast, chondrocytes, Leydig cells, fibroblasts, keratinocytes, astrocytes, placental trophoblasts, and endothelial cells. This cytokine contributes to the differentiation and activation of macrophages, T-cells, and B-cells, and regulates immune responses through its effects on CD4+ T regulatory (Treg) cells, CD8+ Treg cells, and Th17 cells (18). IL-6 signals through two main pathways: classic signaling and trans-signaling. In classic signaling, IL-6 binds to the membrane-bound IL-6 receptor (IL-6R) expressed on hepatocytes and certain leukocytes. In trans-signaling, IL-6 interacts with the soluble IL-6 receptor (sIL6-R) present in the circulation. IL-6 can exert both beneficial and detrimental effects during early pregnancy and in inflammatory diseases. Appropriate levels of IL-6 level are essential for the early immune response against pathogens, including *T. gondii*. Moreover, IL-6 supports embryo implantation and pregnancy maintenance by contributing to placental morphogenesis and promoting embryonic development (19).

Genetic variability in the IL-6 gene variability can influence its expression and may lead to abnormal immune responses (20) potentially contributing to recurrent pregnancy loss (1). The *IL-6* promoter polymorphism -174 G/C has been reported to affect circulating *IL-6* protein levels. Specifically, the C allele is associated with lower *IL-6* levels in the blood, whereas the G allele is linked to higher IL-6 levels (21). Two additional promoter polymorphisms, −597 G/A and −572 G/C, are located within regulatory sequences near the transcription start site. Variations in this region have been demonstrated to influence the transcriptional activity of *IL-6* gene. The G alleles at both

-572 G/C and -597 G/A polymorphic sites are associated with high IL-6 level (22). It has been suggested that genetic variations within the *IL-6* gene may alter DNA conformation or modify the binding affinity of transcription factors, thereby affecting *IL-6* protein expression levels (23). Furthermore, IL-6 gene transcription is regulated through cooperative interactions among different single nucleotide polymorphisms (SNPs) within the promoter region (24).

The *IL-6R* gene is located on chromosome 1q21.3, and its polymorphisms may influence receptor function, domain conformation, IL-6 availability, and circulating levels of s*IL-6R*. Polymorphisms in the *IL-6R* gene have been associated with several disorders, including cardiovascular diseases, rheumatoid arthritis (25). One of the most studied *IL-6R* polymorphism is the A/C variant (Asp358Ala), which results in the substitution of aspartic acid with alanine at position 358 of the receptor protein. This polymorphism is associated with increased levels of sIL-6R due to enhanced cleavage of the membrane-bound IL-6 receptor via the ADAM enzyme, which may preferentially recognize alanine over aspartic acid. The resulting sIL-6R can bind to circulating IL-6 and soluble glycoprotein 130 (sgp130), forming a complex that may buffer the systemic activity of IL-6 (26). The *IL-6R* A/C polymorphism may also alter the conformation of the membrane-bound IL-6 receptors and increase the level of sIL-6R in the blood, thereby modulating IL-6 bioavailability and influencing immune responses to infection (27). To date, no published studies have examined the association between genetic variations within the highly polymorphic promoter region of the *IL-6* gene and circulating IL-6 levels in pregnant women infected by *T. gondii*. Although *IL-6R* gene variants have been investigated in relation to other diseases (25), their role in *T. gondii*-infected women with recurrent pregnancy losses remains unclear. Therefore, the present study was conducted to investigate genetic diversity in the *IL-6* and IL6R genes, as well as circulating IL-6 levels in *T. gondii*-infected women with RPL.

MATERIALS AND METHODS

Inclusion and Exclusion Criteria

The study included women of reproductive age who were infected with *T. gondii* and had a history of pregnancy complications, as well as healthy pregnant women who served as controls. Women outside the reproductive age range and those diagnosed with other chronic infections or autoimmune diseases were excluded from the study.

Study Area and Design

This study included a total of 163 women of reproductive age, comprising 83 *T. gondii*-infected women (Group A), and 80 healthy pregnant women who served as the control group (Group B). Blood samples were collected from all the participants, and placental tissue samples were obtained from 83 infected women. The samples were collected by gynecologists from women attending hospitals in Dir and Peshawar, Khyber Pakhtunkhwa, Pakistan. The infected women (Group A) were further divided into two subgroups: A1 (*T. gondii* antibodies and DNA positive; n=43), and A2 (*T. gondii* antibodies positive and DNA negative; n=40).

Ethical Approval

All participants were informed about the purpose of the study in their native language, and samples and data were collected only after obtaining written informed consent. The study was formally approved by the Research Ethics Board, University of Peshawar, Khyber Pakhtunkhwa, Pakistan, (Registry No. REB-04/02-2024).

Samples and Data Collection

Demographic information, including age, contact with cats, education status, and trimester of pregnancy loss were collected using a predesigned questionnaire. Blood

samples (3mL each) were collected from 163 study participants by trained healthcare workers and gynecologists at the time of pregnancy loss for cases and at delivery for controls. The blood samples were centrifuged at 1500 rpm for 10 minutes, after which sera were separated and stored at -70°C until further analysis. In addition, placental tissue samples were collected from the 83 *T. gondii*-infected pregnant women by trained healthcare workers and gynecologists to confirm the presence of *T. gondi* DNA.

Enzyme-Linked Immunosorbent Assay

T. gondii antibodies were detected by using an ELISA kit (Bio Check, Inc. USA). Serum *IL-6* levels were measured using the Invitrogen Human IL-6 ELISA Kit (Catalog No. BMS213-2) according to the manufacturer's instructions. The assay sensitivity (the lower limit of detection) was 0.92 pg/mL, with standards ranging from 1.56 to 100 pg/mL. The intra-assay coefficient of variation (CV) was 3.4%, while the inter-assay CV was 5.2%. Validation studies demonstrated spike recovery rates ranging from 78% to 105% (mean approximately 88%), whereas dilution parallelism recoveries recoveries ranged from 98% to 111% (mean approximately 105%).

DNA Extraction

DNA was extracted from the blood samples of 80 healthy control women and from the placental tissues of 83 infected women using the Gene JET Gel Extraction Kit (Thermo Fisher Scientific, Catalog No. K0692) according to the manufacturer's instructions. The extracted DNAs were stored at −20°C for further molecular analysis.

Detection of T. gondii DNA

DNA extracted from the placental tissues of infected pregnant women was amplified for the highly conserved B1 gene of *T. gondii* using conventional PCR according to the protocol described by Sardarian et al. (28). The primers used were forward 5'-TCAAGCAGCGTATTGTCGAG-3' and reverse 5'-CCGCAGCGACTTCTATCTCT-3'. The PCR reaction mixture (25µL total volume) contained 12.5 µL of Master Mix buffer (2X, Ampliqon, Denmark), 0.8µL of each primer, 8.9µL of distilled water, and 2µL of template DNA. PCR amplification was performed in a Thermal Cycler (Cell Bioscience, USA) under the following conditions: an initial denaturation at 95°C for 5 minute, followed by 35 cycles of denaturation at 95°C for 25 seconds, annealing at 62°C for 40 seconds, and extension at 72°C for 40 seconds, with a final extension at 72°C for 5 minutes. The amplified PCR product (287bp) was analyzed on a 2% agarose gel and visualized using a gel documentation system (Fig. 1a).

Amplification and Detection of IL-6 and IL-6R Genes

The polymorphic promoter region of the *IL-6* gene was amplified by conventional PCR using the primers and protocol described by Parveen et al. (29). The primer sequences used for *IL-6* gene were: forward 5'-GGAGTCACACACTCCACCT-3', and reverse 5'-GTGGGGCTGATTGGAAACC-3'. The PCR reaction mixture (25 µL) consisted of 12.5µL master-mix buffer (2X), 1µL of each primer, 2µL of template DNA, and distilled water to complete the final volume. The PCR conditions included 1 cycle at 94°C for 3 minutes, followed by 38 cycles at 94°C for 30 seconds, annealing at 64°C for 30 seconds, and extension at 72°C for 60 seconds, and one cycle at 72°C for 7 minutes. The *IL-6R* gene was amplified following the protocol described by Garber et al. (26). The primer sequences used were: forward 5`-GAGGGGAAGGTTCCTTTGAG-3, and reverse 5`-CATGGCATGCTTTTTGTAGC-3`. The PCR reaction mixture (25 µL) contained 12.5µL master mix buffer (2X), 1uL of each primer, 2uL of template DNA, and distilled water to reach the final volume. The PCR conditions consisted of 1 round at 94°C for 5 minutes, 35 rounds at 94°C for 30 seconds, 61°C for 30 seconds, 72°C for 90 seconds, with a final extension at 72°C for 10 minutes.

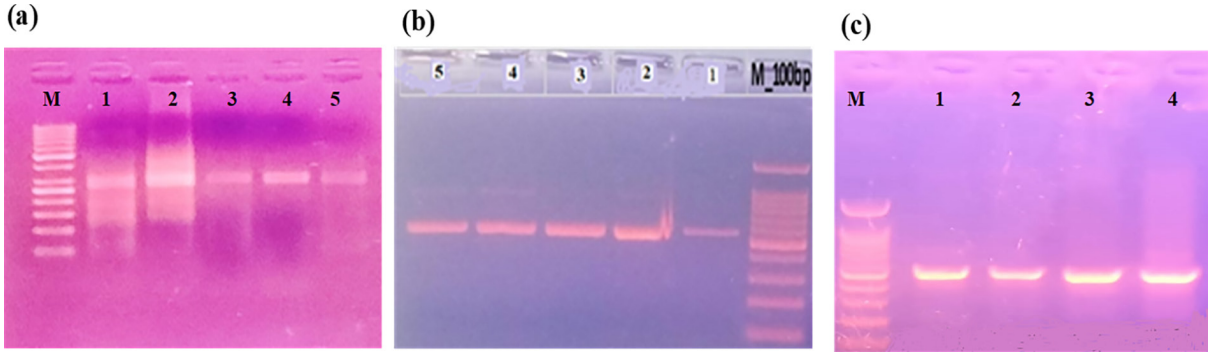

Fig. 1. Gel electrophoresis of amplified DNA products. M=DNA marker. Lanes 1–5 represent amplified samples. a) Amplification of the Toxoplasma gondii B1 gene showing a product size of 287 bp(M=50 bp) b) Amplification of the *IL-6* gene promoter region showing a product size of 533 bp (M=100 bp) c) Amplification of the *IL-6* receptor gene showing a product size of 491 bp (M=100 bp)

The amplified PCR products (*IL-6* gene=533bp, *IL-6R* gene=491bp) were separated by electrophoresis on a 2% agarose gel and visualized using a gel documentation system (Fig. 1b, and 1c).

DNA Sequencing

The amplified products of the *IL-6* and *IL-6R* genes were sequenced using the Sanger sequencing method.

Sequence Analysis

The obtained sequences were analyzed using the Basic Local Alignment Search Tool(BLAST) for sequence confirmation. Multiple sequence alignments and sequence variation analyses were performed using BioEdit software for Windows.

Data Analysis

The latest version of SPSS software was used for statistical analysis, and a P value of less than 0.05 was considered statistically significant. The genotypes and allele frequencies of *IL-6* and *IL-6R* genes were calculated and tested for Hardy Weinberg equilibrium using the Chi-Square test. One-way ANOVA was used to determine and compare the means of continuous variables.

RESULTS

Demographic Data

The ‘ mean age (Mean±SD) of participants was comparable across all groups, with no significant differences (p=0.115). Women in the infected group (Group A) were 4.4 times more likely to have contact with cats compared with healthy pregnant women in the control group (Group B; OR=4.43, 95% CI=2.29–8.58, p<0.001). A significant difference was observed between the groups with respect to educational status, categorized as low education (illiterate, primary, middle) and high education (Higher level) (p=0.004). Women in Group A were 2.7 times more likely to have a lower level of education compared with women in Group B (OR=2.67, 95% CI=1.35–5.29). Furthermore, women in subgroup A1 experienced fewer pregnancy losses during the first trimester compared with women in subgroup A2 (OR=0.33, 95% CI=0.13–0.84, p=0.02, Table 1).

Prevalence of T. gondii Antibodies and DNA

A significant difference was observed between the infected subgroups regarding the prevalence of antibodies (p<0.001). In Group A1, 40 (93.02%) of the infected women were positive for IgM, and 3 (6.98%) were positive for IgG. *T. gondii* DNA was detected by PCR in all the women in Group A1. In contrast, in Group A2, all infected women (n=40) were positive only for IgG, and no *T. gondii* DNA was detected (Table 2).

Genotype and Allele Frequencies of IL-6 Gene Promoter Polymorphisms

The *IL-6* gene, its polymorphic promoter region, the position of transcription factor

Table 1. Demographic Data of the Study Population

Variables		Group A (n=83)		Group B (n=80)	OR (95% CI), *p*-value
		Group A1, (n=43)	Group A2, (n=40)		
Age (Y)	Mean±SD	32.35±9.65	30.24±7.77	29.28±8.55	0.115
Contact with cats	Yes	29 (67.4)	24 (60)	23 (28.8)	4.43 (2.29–8.58), $p<0.001$
	No	14 (32.6)	16 (40	57 (71.2)	
Education	Illiterate	19 (44.2)	13 (32.5)	11 (13.8)	2.67 (1.35–5.29), $p=0.004$
	Primary	09 (20.9)	07 (17.5)	19 (23.8)	
	Middle	08 (18.6)	09 (22.5)	16 (20.0)	
	Higher	07 (16.3)	11 (27.5)	34 (42.4)	
Pregnancy Loss	1st Trimester	10 (23.3)	19 (47.5)	00 (00)	0.33 (0.13–0.84, $p=0.02$)
	2nd Trimester	13 (30.2)	10 (25)	00 (00)	
	3rd Trimester	20 (46.5)	11 (27.5)	00 (00)	

$p<0.05$ is significant, S.D:Standard Deviation

Table 2. Prevalence of *T. gondii* antibodies in the infected pregnant women

Group A1 (n=43)				Group A2, (n=40)		*p*-value
IgM (N)	IgM (%)	IgG (N)	IgG (%)	IgG (N)	IgG (%)	
40	93.02	03	6.98	40	100	<0.001

$p<0.05$ was significant

binding sites, and the amplified gene sequence obtained in this study are illustrated in Fig. 2. Genotyping of the *IL-6* gene promoter region was carried out by direct sequencing of PCR products. The polymorphisms detected in the *IL-6* gene promoter region included -174 G/C (rs1800795), -572 G/C (rs1800796), and -597 G/A (rs1800797). Representative chromatograms showing the identified genotypes of all *IL-6* promoter SNPs are presented in Fig. 3. The Hardy-Weinberg equilibrium for genotypic and allelic frequencies was assessed using the chi-square test. The -174G/C polymorphism showed significant variation in both genotypic ($p=0.005$) and allelic ($p<0.001$) frequencies among the study groups. The -174GG genotype was more prevalent in Group A2 and Group B with frequencies of 47.5% and 56.2%, respectively, whereas it was less frequent in the Group A1, (27.9%). The prevalence of the -174GC genotype was 23.3% in Group A1, 30% in Group A2, and 26.3% in the Group B. The prevalence of the -174CC genotype was 48.8% in Group A1, 22.5% in Group A2, and 17.5% in Group B. The CC genotype was more frequent in Group A, and showed a significant association with infection and pregnancy loss (OR=2.67, 95% CI=1.28–5.54). The rare-174C allele was significantly more common among pregnant women in Group A1, with a prevalence of 60.5%. In contrast, the -174G allele was highly prevalent in Group A2 and Group B, with frequencies of 62.5% and 69.4%, respectively. The C allele was observed approximately 2.2 times more frequently in Group A compared with Group B, suggesting a possible association with increased susceptibility to the disease (OR=2.21, 95% CI=1.41–3.45). No significant difference was found in the genotype frequencies of the -572G/C SNP among the study groups (OR=0.76, 95% CI=0.39–1.46), $p=0.143$). The prevalence of the -572 GG, GC, and CC genotypes was 44.2%, 23.2%, and 32.6% in Group A1; 42.5%, 30%, and 27.5% in Group A2; and 53.8%, 10%, and 36.2% in Group B, respectively. The frequencies of the -572G allele were 55.8%, 57.5%, and 58.8%, whereas the frequencies of the -572C allele were 44.2%, 42.5%, and 41.2% in Group A1, Group A2, and Group B, respectively. The

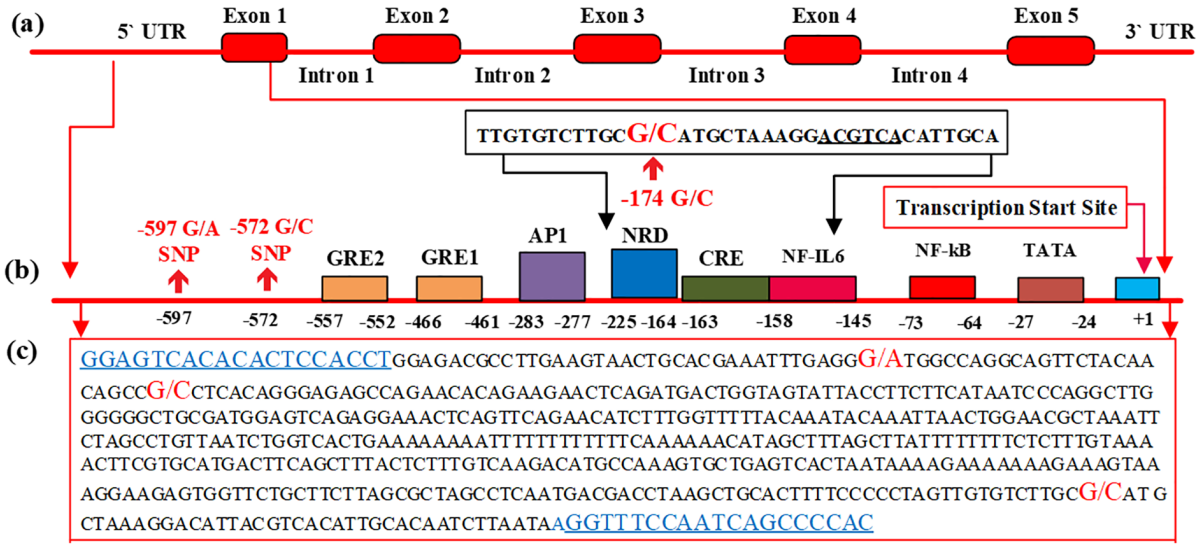

Fig. 2. Structural organization and amplified promoter region of the IL-6 gene analyzed in this study. (a) Intron-Exon structure of the *IL-6* gene. (b) Amplified 5`-flanking region of the IL-6 gene showing transcription factor binding sites, including NF-kB (Nuclear Factor Kappa-B), NF-IL6 (Nuclear Factor IL6), CRE (cAMP Responsive Element), NRD (Negative Regulatory Domain), GRE1 and GRE2 (Glucocorticoid Responsive Element 1 & 2). The positions of single nucleotide polymorphisms are indicated with arrows and highlighted in red. (c) Nucleotide sequence of the amplified *IL-6* promoter region. The primer binding sites are shown in pink, while the identified SNPs are highlighted in red.

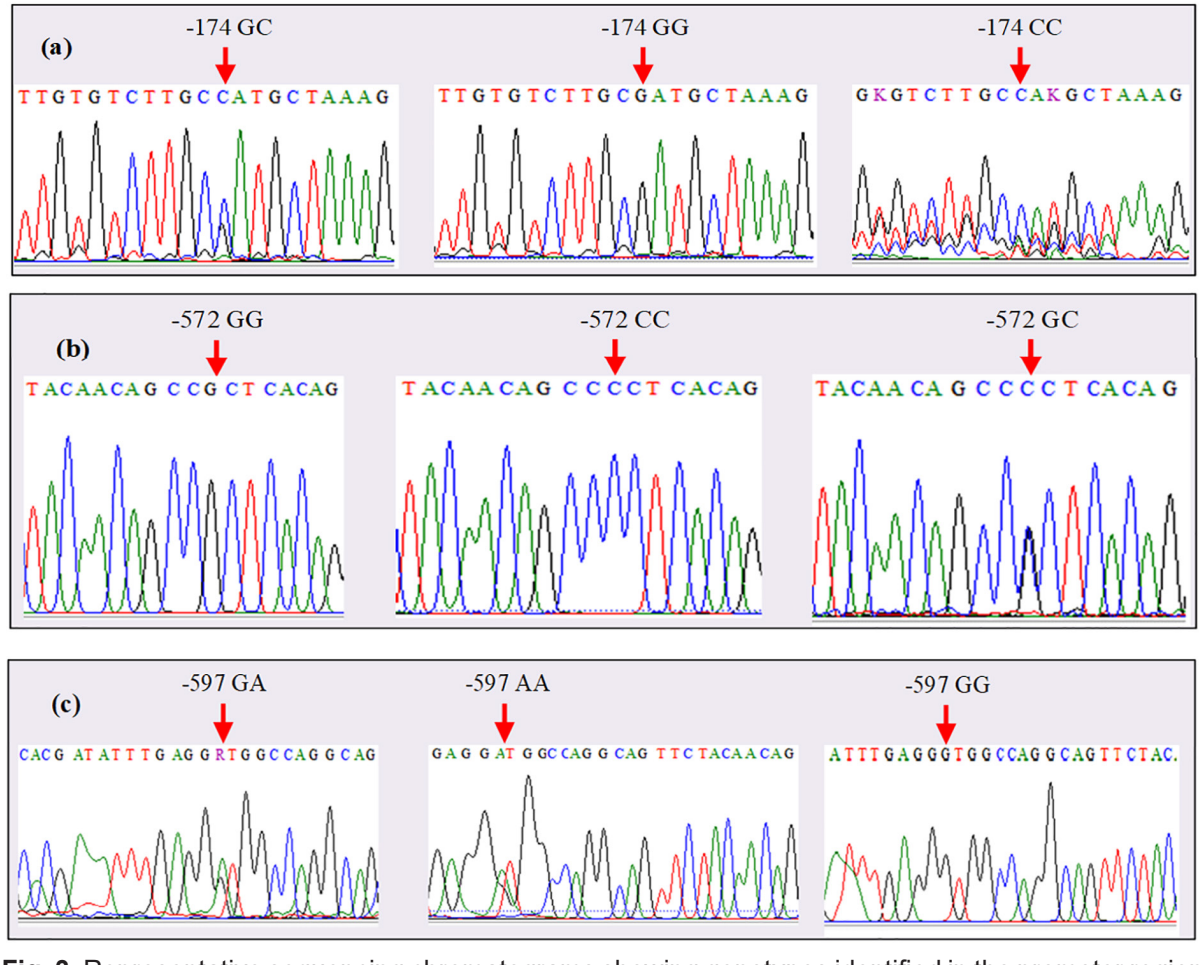

Fig. 3. Representative sequencing chromatograms showing genotypes identified in the promoter region of the *IL-6* gene; (a) -174G/C polymorphism (b) -572G/C polymorphism, and (c) -597G/A polymorphism.

significant difference was observed in allele distribution among the study groups (OR=1.09, 95% CI=0.70–1.70, p=0.429). The genotypic (P=0.0012) and allelic (P<0.001) distributions of the -597G/A SNP differed significantly variable among the study groups. The prevalence of the -597 GG, GA, and AA genotypes was 23.2%, 34.9%, and 41.9% in Group A1; 37.5%, 35%, and 27.5% in Group A2; and 60%, 33.8%, and 6.2% in Group, respectively B. The -597AA genotype was significantly more frequent in infected women in Group A compared with healthy controls in Group B (OR=8.05, 95 % CI=2.95–22.2). The -572A allele was highly prevalent among women in Group A1, with a frequency of 59.3%. In contrast, the -572G allele was more common in Group A2 and Group B, with frequencies of 45% and 23.1%, respectively. The A allele –of the 597G/A SNP showed a strong associates with *T. gondii*-infected women in Group A (OR=3.65, 95 % CI=2.24–5.87) Table 3.

Blood IL-6 Levels in the Study Groups

Blood IL-6 levels were analyzed after adjusting for potential confounding factors, including infection status and stage of pregnancy. The normal distribution of blood IL-6 levels around the mean in each study group is presented in Fig. 5. IL-6 concentrations were expressed as Mean±Standard Deviation (Mean±SD). One-way ANOVA was used to compare the mean IL-6 blood levels between *T. gondii*-infected women and healthy controls, and pairwise comparisons were performed using the Tukey post hoc test. The mean blood IL-6 levels differed significantly variable among the study groups (P<0.001). The highest blood IL-6 level was detected in infected women in Group A2 (29.75±6.80 pg/ml), followed by women in Group A1 (12.47±3.87 pg/ml), while the lowest level was detected in healthy controls in Group B (7.3±3.28 pg/ml, Fig. 4).

Table 3. Genotype and allele frequencies of *IL-6* gene promoter SNPs in the study population

IL-6 Promoter SNPs	Group A (n=83)				Group B (n=80)		OR (95% CI)	*p*-value
	Group A1, (n=43)		Group A2, (n=40)					
	No.	%	No.	%	No.	%		
-174 G/C Genotype								
GG	12	27.9	19	47.5	45	56.2	2.67 (1.28–5.54)	0.005
GC	10	23.3	12	30	21	26.3		
CC	21	48.8	09	22.5	14	17.5		
Alleles								
G	34	39.5	50	62.5	111	69.4	2.21 (1.41–3.45)	<0.001
C	52	60.5	30	37.5	49	30.6		
-572 G/C Genotype								
GG	19	44.2	17	42.5	43	53.8	0.76 (0.39–1.46)	0.143
GC	10	23.2	12	30	08	10		
CC	14	32.6	11	27.5	29	36.2		
Alleles								
G	48	55.8	46	57.5	94	58.8	1.09 (0.70–1.70)	0.429
C	38	44.2	34	42.5	66	41.2		
-597 G/A Genotype								
GG	10	23.2	15	37.5	48	60	8.05 (2.95–22.2)	0.0012
GA	15	34.9	14	35	27	33.8		
AA	18	41.9	11	27.5	05	6.2		
Alleles								
G	35	40.7	44	55	123	76.9	3.65 (2.24–5.87)	<0.001
A	51	59.3	36	45	37	23.1		

p<0.05 is significant.

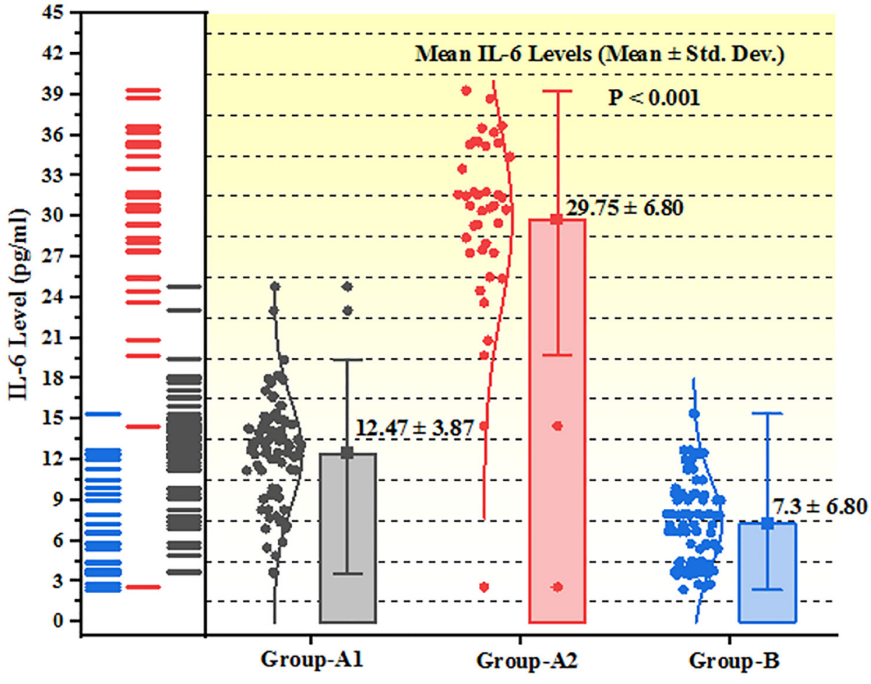

Fig. 4. Distribution of mean serum IL-6 levels among all study groups

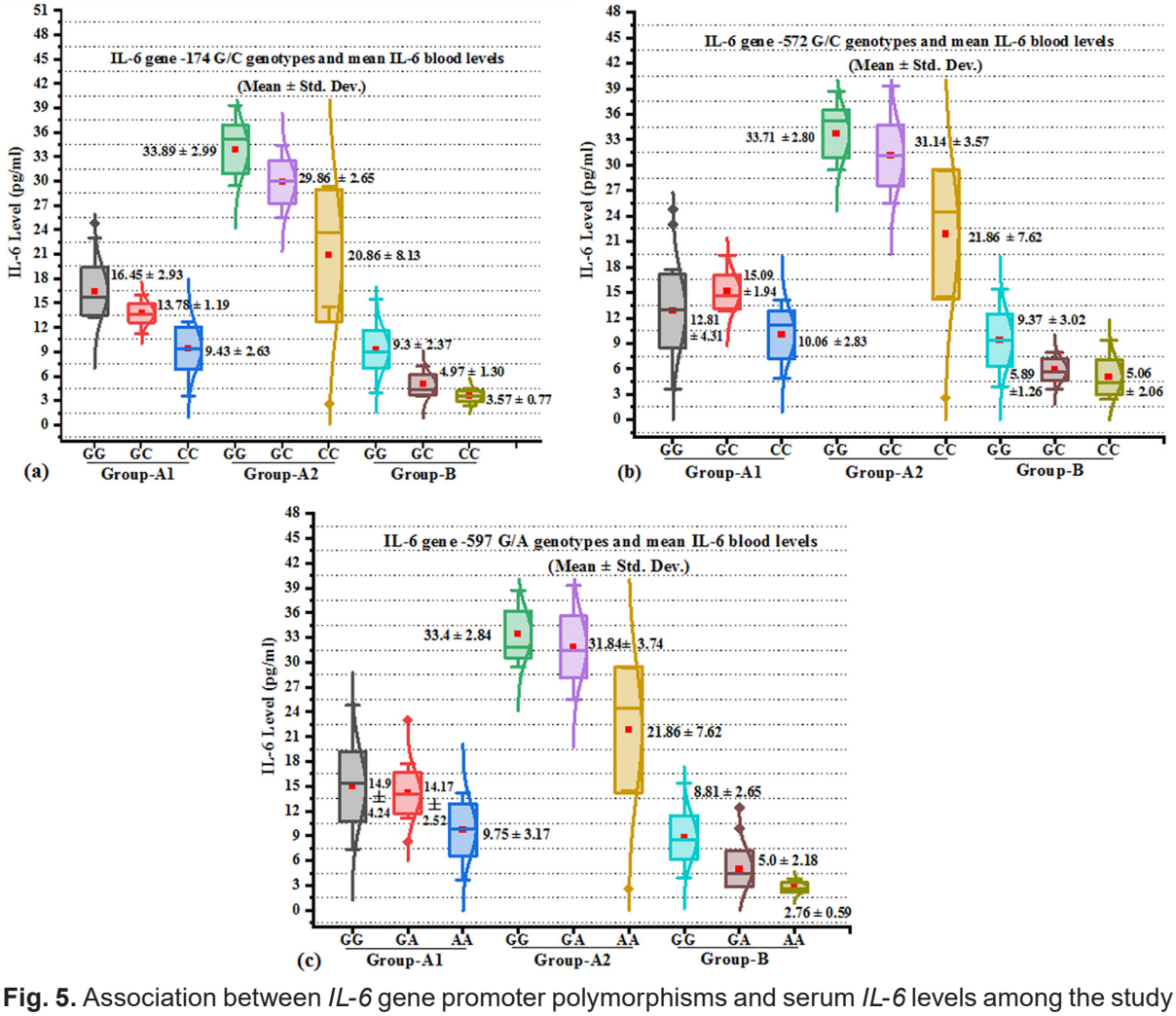

Fig. 5. Association between *IL-6* gene promoter polymorphisms and serum *IL-6* levels among the study groups: (a) -174G/C polymorphisms (b) -572G/C polymorphisms, and (c) -597G/A polymorphisms

Association of IL-6 Gene Genotypes with Blood IL-6 levels

The relationship between *IL-6* gene -174G/C genotypes and blood IL-6 levels differed significantly among the study groups ($P<0.001$). The -174GG genotype was significantly associated with higher blood IL-6 levels, whereas, the -174CC genotype was significantly associated with lower blood IL-6 levels across all groups. Among women in Group B, no significant difference was found between the blood IL-6 levels of the GC and CC genotypes at -174G/C SNP ($P=0.88$, Fig. 5a). Among pregnant women in Group A1, the -572GC genotype of the *IL-6* gene was associated with higher blood IL-6 levels. In contrast, in Group A2 and Group B, the -572GG genotype was associated with high blood IL-6 levels, whereas the -572CC genotype was associated with low blood IL-*6* levels across all groups. No significant differences in blood IL-6 levels were observed between carriers of the -572GG and -572GC genotypes in Group A1 ($P=0.31$) and Group A2 ($P=0.56$). The difference in blood IL-*6* levels between carriers of the of -572 GC and CC genotypes in Group B women was not significant ($P=0.99$, Fig. 5b). For the *IL-6* gene -597GG and GA genotypes were significantly associated with elevated blood IL-6 levels, whereas the -597AA genotype was associated with low blood IL-6 levels across all study groups. The difference in blood IL-6 levels between the -597GG and GA genotypes was not significant in either Group A1 and Group A2 women ($P=0.69$, $P=0.67$, Fig. 5c).

Genotype and Allele Frequencies of IL-6 Receptor Gene Polymorphism

The genotypic ($p=0.0002$) and allelic ($p<0.001$) frequencies of *IL-6* receptor gene polymorphism differed significantly among the study groups. The frequencies of the *IL-6R* gene AA, AC, and CC genotypes were 34.9%, 14%, and 51.1% in Group A1, 50%, 27.5%, and 22.5% in Group A2, and 68.8%, 18.8%, and 12.5% in Group B, respectively. The CC genotype is significantly associated with *T. gondii* infection and risk of pregnancy loss (OR=4.17, 95% CI=1.88–9.30). The A allele of the *IL-6R* gene was significantly more prevalent in Group A2 and Group B, with frequencies of 63.8% and 78.1%, respectively. In contrast, the C allele of the *IL-6R* gene was highly prevalent among women in Group A1, with a prevalence of 58.1%. The C allele was over three times more frequent in infected women in Group A and increased the risk of infection and pregnancy loss (OR=3.24, 95% CI=1.98–5.29; Table 4. Chromatograms of *IL-6R* gene genotypes are shown in Fig. 6.

DISCUSSION

Recurrent pregnancy loss is a global health challenge among women of reproductive-age due to its multifactorial etiology and the substantial economic burden it places on on health system. *T. gondii* infection and genetic

Table 4. Genotype and allele frequencies of *IL-6* receptor gene A/C SNP in the study population

IL-6 Receptor A/C SNP	Group A, (n=83)				Group B (n=80)		OR (95% CI)	*p*-value
	Group A1, (n=43)		Group A2, (n=40)					
	No.	%	No.	%	No.	%		
Genotypes							4.17 (1.88–9.30)	0.0002
AA	15	34.9	20	50	55	68.8		
AC	06	14.0	11	27.5	15	18.8		
CC	22	51.1	09	22.5	10	12.5		
Alleles								
A	36	41.9	51	63.8	125	78.1	3.24 (1.98–5.29)	<0.001
C	50	58.1	29	36.2	35	21.9		

$p<0.05$ is significant.

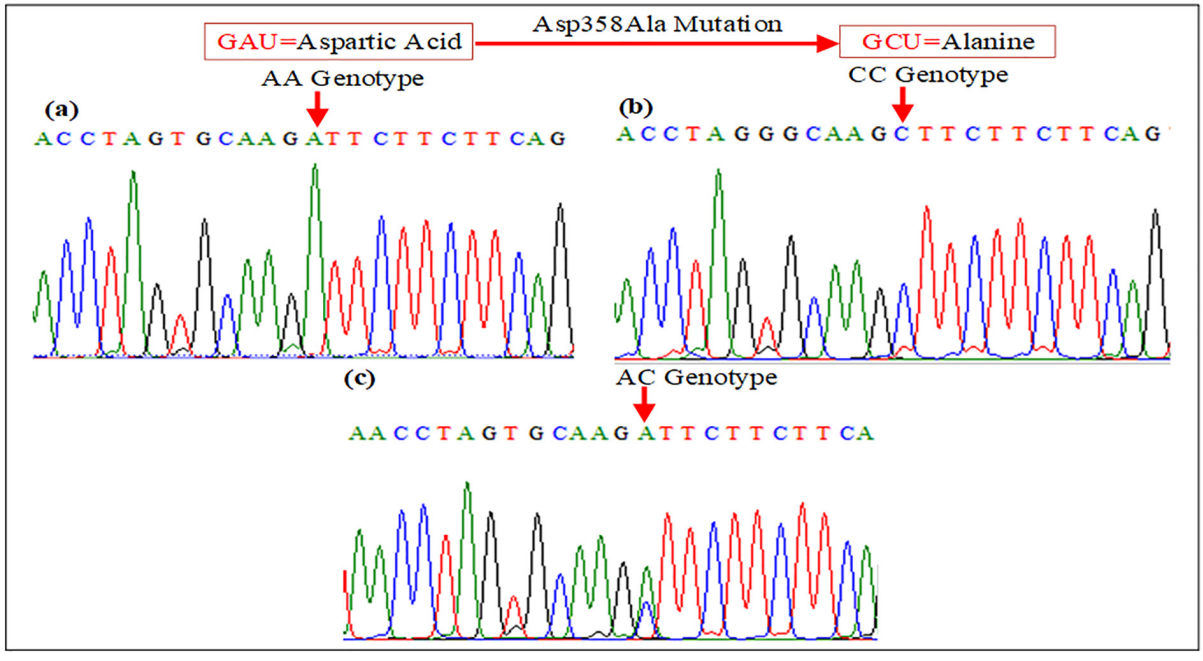

Fig. 6. Representative sequencing chromatograms showing genotypes of the *IL-6* receptor gene A/C polymorphism (Asp358Ala): (a) AA genotype, (b) CC genotype, and (c) AC genotype.

variation in *IL-6* may contribute to pregnancy loss in affected women. systems. To the best of our knowledge, this study is the first to a investigate the genetic diversity of the *IL-6* and *IL-6* receptor genes, and blood *IL-6* levels in *T. gondii* infected women with a history of recurrent pregnancy loss. Our findings indicate that genetic polymorphisms in the *IL-6* and IL6R genes play a significant role in immune regulation and pregnancy outcomes. . From a demographic perspective, women infected with *T. gondii* showed higher levels of contact with cats and were more frequently illiterate compared with the other study groups. Women in Group A1 experienced the highest rates of pregnancy loss during the third trimester, whereas in Group A2 pregnancy loss occurred more frequently during the first trimester, which is consistent with previous report (30). *T. gondii* infection stimulates the secretion of several cytokines, including IL-6. IL-6 is essential for the maintenance and progression of normal pregnancy and contributes to the cytotoxic activity of natural killer cells as well as the acute phase immune response against pathogens (31). *IL-6* gene expression is influenced by single-nucleotide polymorphisms in its promoter region, which can alter transcription factor binding and consequently modify IL-6 production and inflammatory responses (32). The *IL-6R* A/C variant (rs2228145) has been associated with increased levels of soluble *IL-6R* and results in an amino acid substitution (Asp358Ala) that affects mRNA splicing and protein conformation (27). Furthermore, the severity of ocular infection has been linked to altered cytokine expression, and significant retinal inflammation has been observed in *IL-6* knockout mice compared with wild-type mice (18). The increased severity of the infection observed in IL-6–deficient mice is consistent with the findings of the present study, where pregnant women with lower blood IL-6 levels appeared to be more susceptible to *T. gondii* infection. Moreover, the association of genotypes linked to reduced IL-6 production and *T. gondii* infection in pregnant women suggests that genetic variations in the *IL-6* promoter region may influence immune responses in *T. gondii*-infected pregnant women.

In this study, -174G/C polymorphism was significantly associated with *T. gondii* infection in pregnant women. The -174CC genotype, which is linked to lower blood IL-6

levels, was significantly more frequent among infected women in Group A1. In contrast, the -174GG genotype, associated with higher blood IL-6 levels, was more prevalent in women in Group A*2* and Group B. These findings demonstrate a significant association between the *IL-6* gene -174CC genotype, reduced IL-6 levels, and *T. gondii* infection, which is consistent with previously reported reports (31). Shaswati et al. (33) reported that the C allele at the -174 G/C SNP can affect transcription factor binding and is associated with lower blood IL-6 levels. In the present study, the -572G/C SNP was not significantly associated with T. gondii infection or RPL in women. This finding aligns with Demirturk et al. (34), who also found no association between the -572G/C SNP and increased risk of RPL. However, in contrast to our results, the -572G/C polymorphism has been reported to be significantly associated with coronary artery disease (35), chronic periodontitis (36), and malnutrition (37). A study conducted in India reported that the -572CC genotype is highly prevalent and associated with elevated blood IL-6 levels in patients with deep vein thrombosis (38). In the present study, the genotypic and allelic frequencies of the -572G/C SNP did not differ significantly among the study groups. Although no significant association with infection or RPL was observed, the GG and GC genotypes were associated with relatively higher blood IL-6 levels compared with the CC genotype. The result of this study indicates a strong association of the -597G/A polymorphism with *T. gondii* infection, as well as altered blood IL-6 levels in women with RPL. The genotypic and allelic frequencies of the -597G/A polymorphism differed significantly among all study groups. To date, there are no published studies specifically investigating the association between the *IL-6* -597G/A polymorphism and *T. gondii* infection. In contrast, Sharma et al. (38) reported no association between the -597G/A polymorphism and deep vein thrombosis. However, the IL6 -597G/A variant has been associated with c altered blood IL6 levels and has been identified as a potential risk factor in several diseases, including rheumatoid arthritis, and cervical cancer (39). It has been reported that individuals carrying the -597GG genotype exhibit significantly higher blood IL-6 levels compared with those with the GA and AA genotypes (40). The findings of the present study are consistent with these earlier reports, as women with the GG genotypes showed higher blood IL-6 levels than those with GA and AA genotypes, indicating a strong association between the GG genotype and elevated blood IL-6 concentrations. Furthermore, our results demonstrate that the -597AA genotype is strongly associated with lower blood IL-6 levels, and pregnant women with the AA genotype appear to be more susceptible to *T. gondii* infection. The differences in findings among studies may be attributed to variations in ethnicity, age, and the history of chronic infections among study populations. In the present study, blood IL-6 levels differed significantly across all study groups, with the highest IL-6 levels observed in women in Group A2 women. This observation is consistent with the findings of Abdulkhaliq *et al.* (41), who reported significantly elevated blood IL-6 levels in women with recurrent pregnancy loss compared with healthy women. Similarly, Galazios *et al.* (42) demonstrated that women experiencing second-trimester abortion had significantly higher IL-6 concentrations than those with first-trimester abortion.

Previous studies have reported that the *IL-6R* gene A/C polymorphism increases the sIL-6R levels by enhancing the proteolytic cleavage of membrane-bound IL-6 receptor through the action of the ADAM enzyme. The cleavage of membrane-bound IL-6R has been shown to be significantly greater in individuals carrying the *IL-6R* CC genotypes compared with those carrying the AA genotypes at *IL-6R* A/C SNP. The resulting sIL-6R may bind with IL-6 and sgp130 to form a complex that can buffer or regulate the systemic activity of IL-6 (26). In the present study,

the genotypic and allelic frequencies of *IL-6R* gene polymorphism differed significantly among all study groups. The *IL-6R* gene CC genotype was more prevalent among women in Group A1, whereas the AA genotype was more frequently observed in women in Group A2 and the control group (Group B). Webb et al. (43) reported that the *IL-6R* A/C SNP (rs2228145) is associated with approximately twofold higher circulating sIL-6R levels, with CC homozygous individuals exhibiting significantly higher levels than those with AC and AA genotypes. Similarly, Anderson et al. (44) demonstrated that the AC genotype is significantly associated with increased sIL-6R levels compared with the AA genotype. The findings of the present study suggest that the *IL-6R* gene CC genotype is significantly associated with *T. gondii* infection. Pregnant women carrying the CC genotypes at *IL-6R* gene A/C SNP may exhibit elevated levels of sIL-6R, which could buffer systemic IL-6 activity and potentially increase susceptibility to infection.

Study Limitations

This study has several limitations. The relatively small sample size and the inclusion of women only from Pakistan may limit the generalizability of the findings to other populations. Additionally, the serum levels of sIL-6R and sgp130 were not measured, which could have provided further insight into the dynamics of IL-6 signaling. Future studies should include larger, more diverse populations, and the assessment of serum sIL-6R and sgp130 may further clarify the mechanisms linking IL-6 polymorphisms with recurrent pregnancy loss.

CONCLUSION

In conclusion, the *IL-6* gene -174G/C and -597G/A polymorphisms are significantly associated with T. gondii infection and altered blood IL-6 levels. The CC genotype at the -174G/C SNP and the AA genotype at the -597G/A SNP were more prevalent among infected women in Group A1 and were significantly associated with lower blood *IL-6* concentrations. In contrast, the GG genotypes at both the -174G/C and -597G/A polymorphic sites were more common among infected women in Group A2 and the control Group B and were associated with higher blood IL-6 levels. The *IL-6* gene -572G/C polymorphism did not show a significant association with *T. gondii* infection or recurrent pregnancy loss. In addition, the *IL-6R* gene CC genotype was significantly associated with increased susceptibility to *T. gondii* infection in pregnant women. In summary, high *IL-6*-producing alleles were more frequent among infected women in Group A2, whereas low *IL-6*-producing alleles were more prevalent among infected women in Group A1.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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