ORIGINAL ARTICLE

New Horizons for the Relationship between Genetic Variations and Two Immune Markers in Spontaneous Abortion among Iraqi Women: A Cross-Sectional Study

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ABSTRACT

Background: *Spontaneous abortion (SA) is a significant health concern in Iraq. It can be caused by genetic, immunological, and environmental factors.* ***Objective:*** *The aim of this study is to identify the association between CD34+ and HLA-F levels with Toll-like receptors (TLR2, TLR4, TLR7) polymorphisms and their predisposing to SA.* ***Methodology:*** *A survey involving 200 participants was conducted, comprising 100 women diagnosed with SA at the beginning of pregnancy and 100 pregnant women serving as a control group of the same gestational age, all were referred to two Maternity and Children Hospitals in Diyala and Babylon, Iraq. The age range of participants was 20 to 40 years. ELISA was used to quantify the concentrations of immune parameters CD34+ and HLA-F. PCR-RFLP technique was done to evaluate variations in three Toll-like receptor genes.* ***Results:*** *A statistically significant association (p-value ≤0.001) between CD34+ and HLA-F concentrations and a risk factor for SA. The TLR4 (rs1927914) (G/A) genetic variation has revealed a novel association between the genotype GG (2.97±0.7ng/ml) and SA, which have higher levels of HLA-F concentration in peripheral blood of aborted women at a significant value (P-value ≤0.006) compared with other genotypes.* ***Conclusions:*** *The research showed lower CD34+ and HLA-F concentrations in SA women and a novel association between Toll-like receptor 4 genotype GG and elevated HLA-F concentration in Iraqi women experiencing spontaneous abortion and needs further investigations.*

INTRODUCTION

Spontaneous abortion is a prevalent adverse reproductive outcome globally, posing a significant challenge to efforts to promote maternal health [1]. Risk factors include endocrine, anatomical, infectious, genetic, hemostatic, and immunological elements. The diagnosis is complicated by occasionally contradictory guidelines from pertinent international specialist societies [2]. The unidentified cause of recurrent spontaneous abortion hinders therapy and causes patient distress. Immunological disorders may be a fundamental cause of adverse pregnancy outcomes [3]. The guideline recommendations highlighted prognostic factors such as autoantibodies, natural killer cells, regulatory T cells, dendritic cells, plasma cells, and the HLA system's involvement [4]. HLA antibodies during pregnancy can lead to organ transplant rejection and blood transfusion adverse outcomes, as extravillous trophoblasts invade uterine glands, facilitating chemotrophic and histiotrophic nutrition [5]. The mid-secretory endometrium expresses HLA-F, correlated with immune cell infiltration, while extravillous trophoblast cells express HLA-G and HLA-F, interacting with maternal immune cell receptors [6]. Recent discoveries indicate HLA-F may be involved in viral infections, cancer immunity, fertility, and reproduction, highlighting the growing interest in this unexplored HLA class I protein[7].

Endothelial progenitor cells (EPCs) increase during physiological pregnancy, which is crucial for placentation. Their count could be a key indicator for monitoring pregnancies and developing innovative FGR management strategies [8]. A CD34, a transmembrane phosphoglycoprotein, is present in hematopoietic stem and progenitor cells and is associated with bone marrow transplantation, indicating increased progenitor activity [9]. This marker belongs to a single-pass transmembrane protein class. It is expressed in various cells, including hematopoietic progenitor cells, vascular endothelial cells, interstitial precursor cells, and various interstitial tumour cells [10]. Toll-like receptors, essential in the innate immune system, regulate epigenetic elements and are activated by fetal infections and pro-inflammatory stimuli, enhancing uterine contractility and cervical ripening [11]. Pregnancy-induced immunosuppression

triggers TLRs response, maintaining anti-infective resistance in female genital organs. However, an imbalance between TLRs in URSA patients may undermine immune tolerance and cause spontaneous abortion [12]. TLR7 identifies ssRNA viruses, initiates antiviral and inflammatory responses, and genetic polymorphisms may affect infection progression. SNPs in TLR4 may be markers for recurrent spontaneous miscarriage [13]. Our study investigates the link between genetic alterations in three genes of TLRs and CD34+ and HLA-F levels and their association with spontaneous abortion incidence, offering a new approach to abortion researches.

METHODOLOGY

Ethical Approval

Part of the research was derived from a doctoral dissertation; the study project was turned in to the Ethics Committee of the College of Medicine Council at the University of Babylon. Following clearance, the Research and Development Committees of the Babylon Health Department for Maternity and Children and the Diyala Health Department were contacted to get permission to conduct the study in hospitals connected to the research issue. Receiving the numbered letter 8199, dated 12 February 2024.

Study Design and Site

A cross-sectional study was conducted from February 15 to November 15, 2024. Samples were obtained from the maternity emergency units of Diyala Hospital and Babylon Maternity and Children's Hospital. Experiments were carried out in the teaching laboratories of Baquba Teaching Hospital in Dibali, encompassing the ELISA and molecular screening units.

Participants and Sample Acquisition

The study participants were categorised into two groups: 100 women who were currently undergoing unexplained spontaneous miscarriage, diagnosed prior to the intervention during the initial months of pregnancy. A comparison was made with 100 pregnant women who had no history of miscarriage, serving as a control group at different stages of pregnancy. All participants provided verbal consent. A study-specific questionnaire was used to collect data from 20-40-year-olds without autoimmune diseases, cervical issues, ectopic pregnancy, or molarian pregnancy. Five millilitres of venous blood were collected using sterilised syringes (Shandong- China) and subsequently divided into two divisions: one placed in a Gel tube (Arth Alrafidain-Iraq) and another in an EDTA tube (Arth Alrafidain-Iraq). The blood samples underwent centrifugation (Hettich – Germany) at 5000 rpm for 5 minutes following gentle mixing. The serum was aliquoted into sterile tubes, which were subsequently frozen at -20°C for later use.

Quantitative ELISA Technique

The concentrations of immune parameters CD34+ and HLA-F anti-Alpha chain in peripheral blood were quantified using the ELISA technique (BioTek – USA), and Kits from (My BioSource-USA), and concentration standards were established to facilitate the extraction of absorbance. This procedure facilitated the derivation of concentrations from absorbance measurements by applying the Beer-Lambert law for both groups. Plates are coated with human CD34 monoclonal antibodies, which bind to the sample's CD34 protein. Streptavidin-HRP binds to biotinylated CD34 antibody, and absorbance is measured at 450 nm. The same method applies to HLA-F concentration as in source [13].

Molecular Detection of Genetic Variations in Toll-like Receptor Genes

Select Primers for the Selected SNPs:

Molecular methods such as the RCR-RFLP technique were used. Three primers were designed specifically for this study after selecting variants from the literature associated with SA. The NCBI-primer BLAST software was developed, and primers were tested in the Thermo Fisher tool as Oligo Calc software, ordered from (Macrogen-LIGO-Korea). Three reference SNP (TLR-2 SNP rs3804100, TLR4 SNP rs1927914, and TLR7 SNP rs179008) genes were chosen, as shown in (Figure (1,2,3).

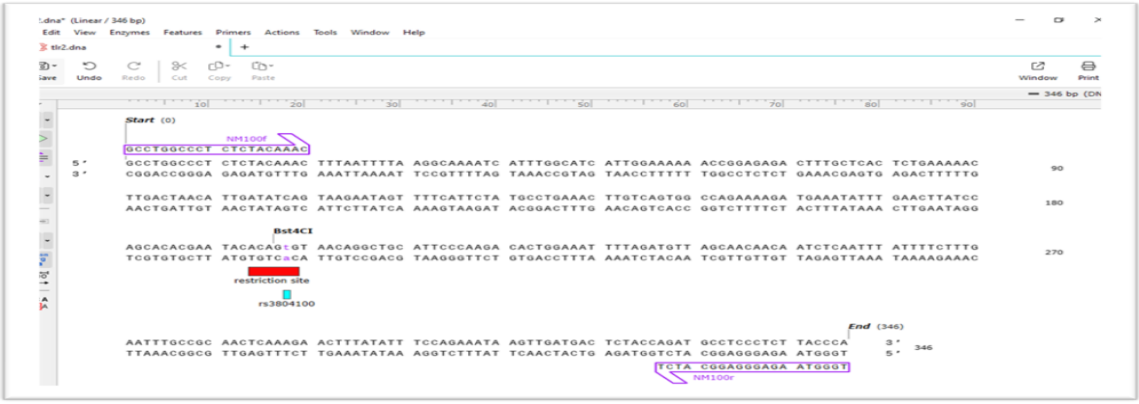

Fig. 1: Pick primer *TLR2 Gene.*

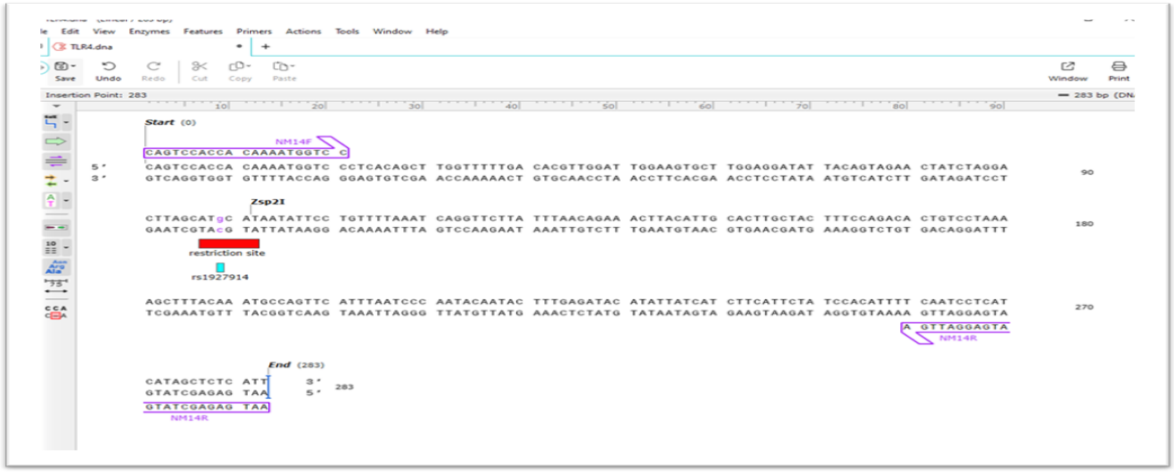

Fig. 2: Pick Primer *TLR4 Gene*

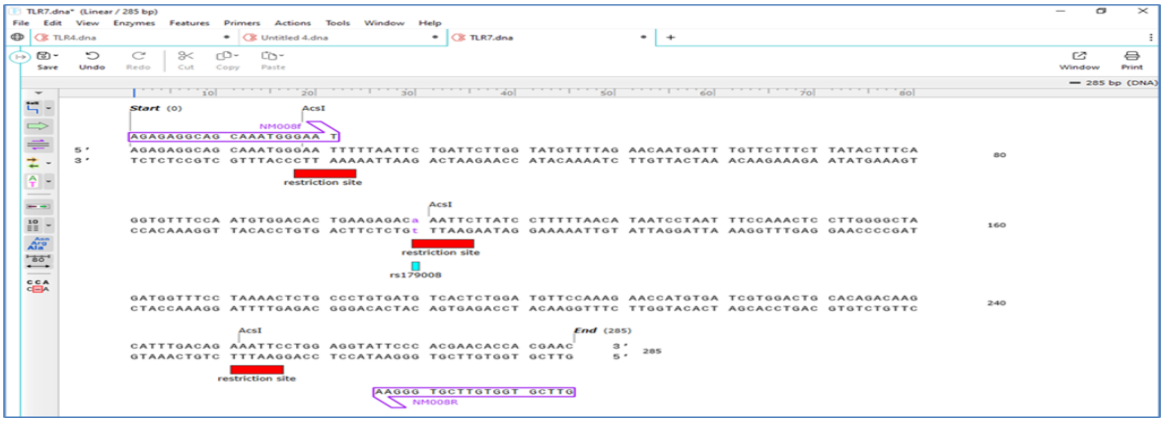

Fig. 3: Pick Primer *TLR7 Gene*

Selected the Appropriate Restriction Enzymes:

The restriction enzymes were selected from the Snap-Gene viewer software (V6.0.5) as (Bst4C I, Zsp2 I, and Acs I) and used per the instructions of the (Sib Enzyme- Russia) manufacturer.

Procedure

First, blood samples were thawed and mixed, and DNA was extracted using the salting-out method, as in Hashim & Al-Shuhaib [14]. Concentration and purity were determined through spectrophotometric analysis. Then, samples were frozen until ready to use. Second, the extracted DNA for three TLRs genes (2,4,7) was amplified using a Thermo-Cycler, followed by processing five samples at varying annealing temperatures. The optimal band was identified using electrophoresis in agarose. The DNA was mixed with PCR Master Mix components, following the manufacturer's instructions (Cynthol-Russia). Third, the amplified DNA for each TLRs (2,4,7) gene was combined with specific restriction enzymes (Bst4C at 65℃, Zsp2 I at 60℃ and Acs I at 50℃) from Sib Enzyme-Russia, following the manufacturer's instructions for incubation. Finally, DNA was electrophoresed on a 2% agarose gel, stained with ethidium bromide dye, and compared to a DNA ladder. Genetic patterns were read using UV light-transilluminators.

Sequencing of PCR Amplified Products

The design of the PCR-RFLP experiment was validated by sequencing three genotypes of each gene, demonstrating complete compatibility at 100%. The resolved PCR amplicons underwent forward and reverse sequencing following the protocols specified in the instruction manuals from the sequencing company, Macrogen Inc., located in Geumchen, Seoul, South Korea.

Statistical Analysis

The study utilised SPSS version 28 for statistical analysis, concentrating on the comparative assessment of variables between pregnant women and those who have experienced abortion. Independent T-tests were utilised to examine the differences in levels of two markers across two groups and the associations between genotypes and abortion risk, with a significance threshold at $p \leq 0.001$.

RESULTS

Association Between CD34+ and HLA-F Marker Levels in Both Groups

The independent T-test results indicate a significant relationship (p-value ≤0.001) between CD34+ and HLA-F concentrations, which were significantly elevated in healthy pregnant women during the third trimester, recorded at 8.05±1.77 and 4.54±1.50 ng/ml, respectively, within the range of 7.0-9.0. The levels in women who experienced miscarriages were lower, recorded at (1.54±0.34) and (2.65±0.56) ng/ml during the first trimester, within the range of (2.0-4.0) as shown in (Figure 4).

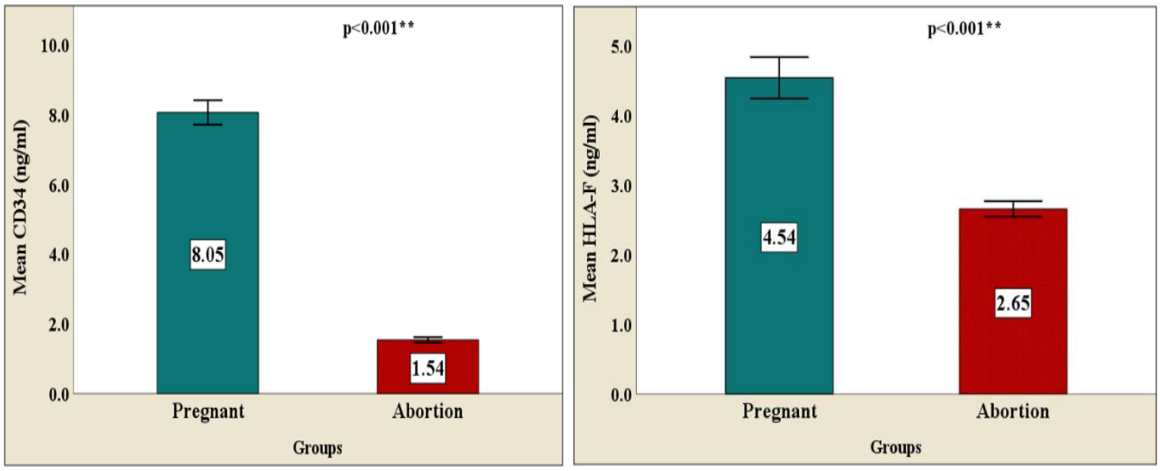

Fig. 4: Concentration of CD34+ (ng/ml) and HLA-F (ng/ml) in the Two Analyzed Groups

Identification of Single Nucleotide Polymorphisms in the TLRs (2,4,7) Genes Across Both Groups

The PCR-RFLP technique was used to identify a single nucleotide polymorphism in pregnant and aborted women, revealing three distinct genotypes in TLR2 (rs3804100) gene T>C: TT, TC, and CC. The wild-type homozygote genotype amplified the T allele at a product size of 196+150 bp and the C allele at 346 bp. The product size of the TLR4 (rs1927914) gene in Promoter chr9:117702447 (GRCh38.p14) Alleles G>A genotypes were GG, GA, and AA at the locus varies, with wild-type homozygous amplification of A at 283 bp and mutant-type homozygous amplification of G at 101+182 bp. The TLR7 (rs179008) Gene A>T SNP gene polymorphism in miscarried and pregnant women reveals three genotypes: AA, AT, and TT. The A allele has a product size of 18+35+91+141 bp, while the mutant-type homozygote genotype amplifies the T allele at a product size of 18+35+232 bp (Figures (5,6,7).

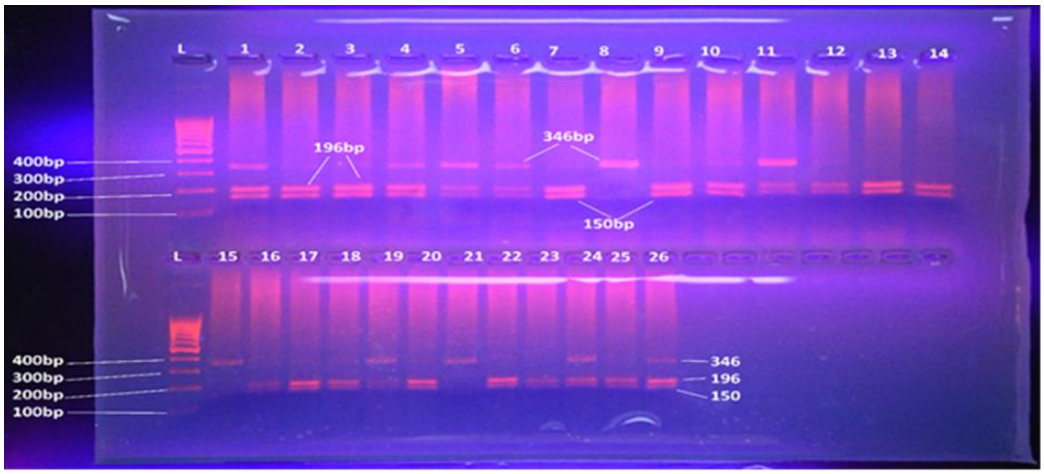

Fig. 5: Genotyping of *TLR2 (rs3804100)* 346bp gene by PCR-RFLP, restriction product resolved on 2% agarose, lane L 100 bp DNA ladder: CC genotype lanes 8,15 and 21; TC genotype lanes 1,4,5,6,11,19,24 and 26; TT genotype lanes 3,7,9,10,12,13,14,16,17,18,20,22.

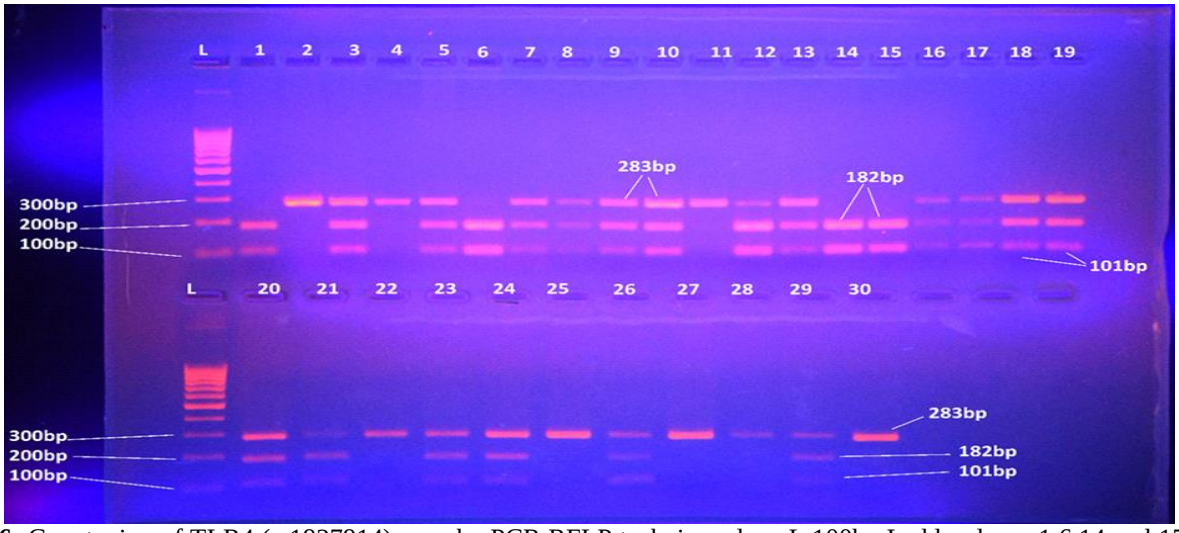

Fig. 6: Genotyping of TLR4 (rs1927914) gene by PCR RFLP technique, lane L 100bp Ladder; lanes 1,6,14 and 15 GG genotype; lanes 3,5,8,9,10,11,12,16,17,18,19,20,21,23,24,26,29 GA genotype; lanes 2,4,11,22,25,27,28 and 30 AA genotype.

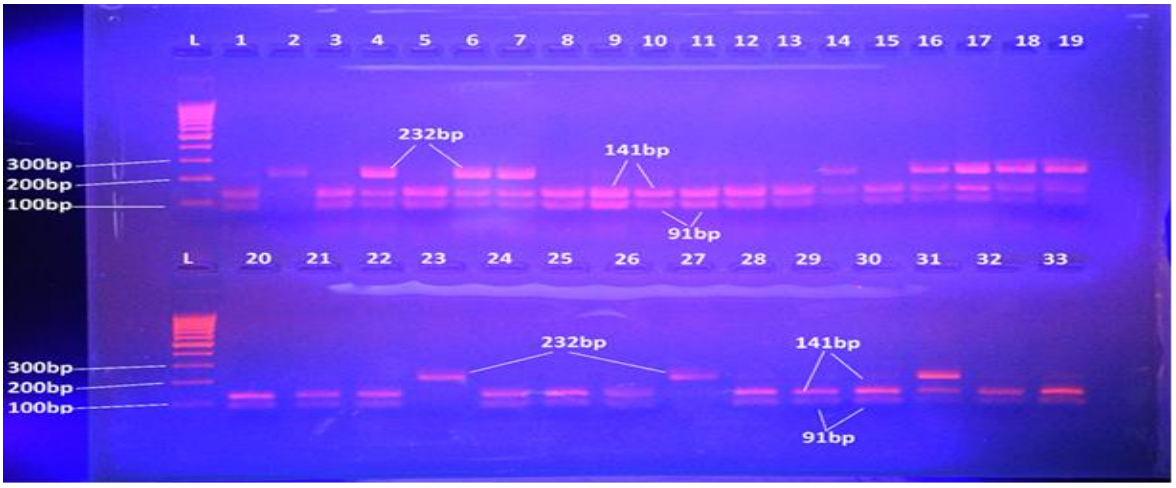

Fig. 7: Genotyping of *TLR7(rs179008)* by PCR-RFLP technique, lane L 100bp DNA ladder; lanes 2,23, and 27 TT genotype; lanes 4,6,7,17,16,17,18,19 and 31 AT genotype; other lanes AA genotype.

Effects of Genotypes TLR2 (rs3804100) (T/C) SNPs on Marker levels in Studied Groups

The T-test analysis shows no significant difference in (p-value > 0.05) between TLR2 rs3804100 genotypes and CD34+ and HLA-F concentrations in aborted and pregnant women, as shown in (Table 1).

Effects of Marker Levels in Studied Groups on Genotypes TLR4 (rs1927914) (G/A) SNP

The study found no significant difference in CD34+ concentration between women who experienced abortion and those healthy pregnant, but a notable difference (p-value ≤0.05) in HLA-F concentration with the GG genotypes (2.97±0.7ng/ml) in aborted women (Table 2).

Impact of TLR7 rs179008 (A/T) SNP Genotypes on Biomarker Levels in Analysed Groups

The T-test analysis shows no significant difference (p-value > 0.05) between the genotype TLR7 polymorphism gene and CD34+ and HLA-F concentrations in aborted women and pregnant individuals (Table 3).

Table 1: Effects of Genotypes TLR2 SNPs on Marker Levels in Studied Groups

Groups	TLR2	N	CD34+ (ng/ml)		HLA-F (ng/ml)	
			Mean±SD	p-value	Mean±SD	p-value
Pregnant	TT	63	7.89±1.79	0.438 Ns	4.49±1.42	0.225 Ns
	TC	30	8.39±1.69		4.41±1.43	
	CC	7	8.06±1.92		5.47±2.27	
Abortion	TT	57	1.57±0.38	0.470 Ns	2.59±0.62	0.678 Ns
	TC	39	1.49±0.29		2.7±0.5	
	CC	4	1.60±0.11		2.62±0.27	

NS: non-significant

Table 2: Effects of Genotypes TLR4 SNPs on Marker Levels in Studied Groups

Groups	TLR4	N	CD34 (ng/ml)		HLA-F (ng/ml)	
			Mean±SD	p-value	Mean±SD	P-value
Pregnant	AA	29	7.9±2.05	0.281 Ns	4.59±1.64	0.797 Ns
	GA	60	8.25±1.53		4.52±1.38	
	GG	11	7.38±2.12		4.48±1.82	
Abortion	AA	41	1.52±0.32	0.976 Ns	2.47±0.57	0.006*
	GA	41	1.55±0.33		2.66±0.41	
	GG	18	1.58±0.42		2.97±0.7	

* Significant differences at p-value ≤0.05

Table 3: Effects of Genotypes TLR7 SNPs on Marker Levels in Studied Groups

Groups	TLR7	N	CD34 (ng/ml)		HLA-F (ng/ml)	
			Mean±SD	P-value	Mean±SD	P-value
Pregnant	AA	42	8±1.68	0.849 Ns	4.4±1.77	0.746 Ns
	AT	49	8.05±1.84		4.63±1.24	
	TT	9	8.37±1.92		4.63±1.45	
Abortion	AA	67	1.55±0.35	0.676 Ns	2.69±0.62	0.232 Ns
	AT	17	1.58±0.3		2.43±0.44	
	TT	16	1.48±0.34		2.63±0.35	

NS: non-significant

DISCUSSION

The immune system is crucial for pregnancy and embryo implantation, requiring an intricate interplay of immune cells, cytokines, and regulatory pathways to maintain a stable pregnancy [15]. In our work, CD34+ and HLA-F levels in pregnant women were higher in the control group, while their levels diminished in women who experienced SA. This confirms the role of these two factors in maintaining pregnancy safety and continuity. The current study agrees with a study that revealed that the model's precision could be improved by increasing the sample size and considering factors like nutrition, chronic diseases, and stress. Research shows CD34+ cells decrease in pregnancy trimester, re-cloning efficiency, and peripheral blood levels in women with SM [16]. A study reported a significant reduction in maternal peripheral blood CD34+ cells in women with congenital kidney anomalies, suggesting potential for CD34+ cell-based regenerative therapy for vascular, ischemic, and inflammatory disorders. Clinical trials show safety, practicability, and validity, while using a partner's white blood cells for immunisation reduces pregnancy failure [17]. Also, the study indicates that human leukocyte antigen F protein levels are diminished in the extravillous trophoblast and villous placenta of cases with severe early-onset preeclampsia, regardless of the presence of small-for-gestational-age neonates, compared to term and preterm births [18]. The immune system plays a vital role in embryo implantation and pregnancy; however, the molecular intricacies are still debatable. In the last four decades, there has been significant interest in human leukocyte antigens (HLA)-G and -F [19].

In the present study, the analysis of our results showed no statistically significant differences (p>0.05) between *TLR2 rs3804100 gene* and CD34+ and HLA-F concentrations in pregnant and aborted women. This contradicted a study that found two different TLR2 alleles may result in abnormal innate immune responses, potentially contributing to very preterm birth [20]. Another

study found that all participants belong to the Ukrainian population. Consequently, TLR9 and IL-10 SNPs have been identified as significant factors in the occurrence of spontaneous abortion. TLR2, TLR4, IL-6, IL-8, and PGR SNPs were recognised as additional factors that may influence the risk of miscarriage [21]. Miscarriage results are often insignificant, emphasising the need to assess general health, hormone levels, and causes. The model's accuracy may improve by adding nutrition, chronic diseases, and stress to the sample.

In contrast, in our work table (2) demonstrated a significant and noteworthy association (p-value ≤ 0.05) between TLR4 (rs1927914) SNP and the fluctuations in HLA-F concentration within the aborted group, where individuals with the GG genotype exhibited elevated levels of HLA-F, while those with the AA and GA genotypes displayed reduced HLA-F concentrations. This finding is novel, has not been previously examined, and requires more investigation. The HLA class Ib molecules, such as HLA-F, are expressed by extra-villous trophoblast cells and possess immunomodulatory properties, contributing to the feto-maternal interface [22]. Also, a study found that HLA-F protein levels are linked to immune cell infiltration and plasma progesterone concentrations [23]. The overexpression of TLRs may excessively activate the innate immune system mediated by MyD88, which is considered to be related to the pathogenesis of spontaneous abortion [24].

Also, table (3) showed that the study reported no significant correlation between TLR7 polymorphism genotypes (AA, AT, TT) and CD34+ and HLA-F levels in women who have had abortions. In contrast, Numerous studies have demonstrated a correlation between genetic variation in TLR 7 and various diseases, particularly viral infections, as well as its role in spontaneous abortion. However, to our knowledge, there is a lack of research connecting TLR7 variation with CD34+ and HLA-F concentrations. Polymorphisms in TLR7 and TLR9 may modulate the risk of placental infections and pregnancy problems [25]. Also, The TLR7 rs179008A > T ($p = 0.049$) polymorphism was nearly associated with CMV-positive (CMV+) status. Conversely, the interleukin (IL)-6 rs10499563T > C ($p = 0.001$) polymorphisms were linked to CMV negative (CMV-) status [26]. Spontaneous abortion is a multifactorial disease as maternal age and recurrent abortion significantly influence immune system elements. Future research should analyse environmental factors, conduct more extensive studies, consider genetic, psychosocial, and age-related factors, and include diverse age groups.

CONCLUSIONS

The study indicates that CD34+ and HLA-F concentrations were significantly elevated in healthy pregnant women, while levels were lower in women experiencing miscarriages. The study revealed a new and interesting association between genetic variation in Toll-like receptor 4 genotype GG, who had the height of HLA-F concentration in the serum of Iraqi women with spontaneous abortion, which warrants further investigation and research.

Conflict of Interest

We do not have any declared conflicts of interest.

Financial Help

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