

## Review Article

# Immunologic Factors and Genomic Considerations in Recurrent Pregnancy Loss: A Review

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### ABSTRACT

#### Article history

Received: 8 Nov 2023

Accepted: 26 Dec 2023

Available online: 24 Feb 2024

#### Keywords

Fetal-maternal interface

Genomic factors

Immunologic factors

Recurrent pregnancy loss



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Recurrent pregnancy loss (RPL), which is also known as repeated miscarriage, affects around 2-3% of women who are trying to conceive. While some factors contributing to RPL have been identified, the cause of almost half of all cases remains unknown. Immunological factors have been proposed as one of the potential causes of such miscarriages. However, it is hard to track the common factors leading to RPL since the individual genomic identity of aborted fetuses is different in a family like other siblings. The immunological factors involved in the pathogenesis of miscarriage generally result from either the function of the maternal immune system attributable to her genomic background or the fetal origination established by both maternal and paternal genomic backgrounds that constitute the fetal genome.

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## Introduction

Recurrent pregnancy loss (RPL) is commonly defined as three or more consecutive unsuccessful pregnancies before 22 weeks of gestation, affecting approximately 2-3% of women who intend to become pregnant [1]. While severe fetal malformations, caused mainly by chromosomal abnormalities, have been established as the proven factor in RPL, in many cases, no clear cause for RPL can be identified. The other known risk factors for such pathological conditions are infections, Antiphospholipid Antibody Syndrome (APS), anatomical problems, and immunological and genetic factors [2]. According to the literature, 40% to 50% of RPL cases are classified as idiopathic or unexplained RPL with unknown etiology. Various factors have been suggested for these cases, which can be divided into two general groups: environmental factors, such as ovarian aging, unknown alloimmune factors related to the environment, and genetic factors affecting immune system function [3].

In RPL studies, the genetic material from two generations, the mother/father and the offspring, is usually analyzed. This involves examining the expression of genes and proteins in different compartments, namely the mother, placenta, and fetus. Gene/protein expression Aberrations can impact maternal and fetal organisms [4]. According to the Euro-Team Early Pregnancy protocol for RPL, it was suggested to perform a cytogenetic investigation of the products of conception (POC) material if a couple experiences two spontaneous miscarriages and both partners have normal karyotypes. This helps

in providing prognostic information and to assess the efficacy of treatment. However, routine POC genetic testing has not yet been widely adopted due to the challenges in obtaining biological material suitable for *in vitro* culture, a prerequisite for karyotyping [5].

As a result, in the last two decades, various targeted molecular assays have been developed for the detection of aneuploidy from uncultured material, such as fluorescent in situ hybridization (FISH), multiplex ligation-dependent probe amplification (MLPA), and quantitative fluorescent polymerase chain reaction (QF-PCR). Furthermore, with cutting-edge technologies such as array comparative genomic hybridization (aCGH) and single nucleotide polymorphisms (SNP) microarrays, it is now possible to conduct high-resolution profiling of microdeletions/duplications on a genome-wide scale. In addition, next-generation sequencing (NGS) technology offers single base pair resolution and the most sensitive detection of mosaicism among all available methods [6].

### Genetic factors involved in RPL

Thus far, various genetic factors have been identified as underlying causes of RPL. Most studies have categorized genetic factors according to their maternal or paternal source. Nonetheless, despite some factors being passed explicitly from one parent, many can be inherited parallelly from both parents, leading to RPL. Furthermore, the lack of RPL in the siblings of both couples experiencing this issue suggests that the combination of genetic profiles from two families comprising the fetal genomic

background may be responsible for this pathological state. Accordingly, the genetic factors leading to RPL have been reviewed in this study in 4 groups. Figure 1 illustrates these genetic factors and their origins.

**Paralleled maternal/paternal genetic factors**

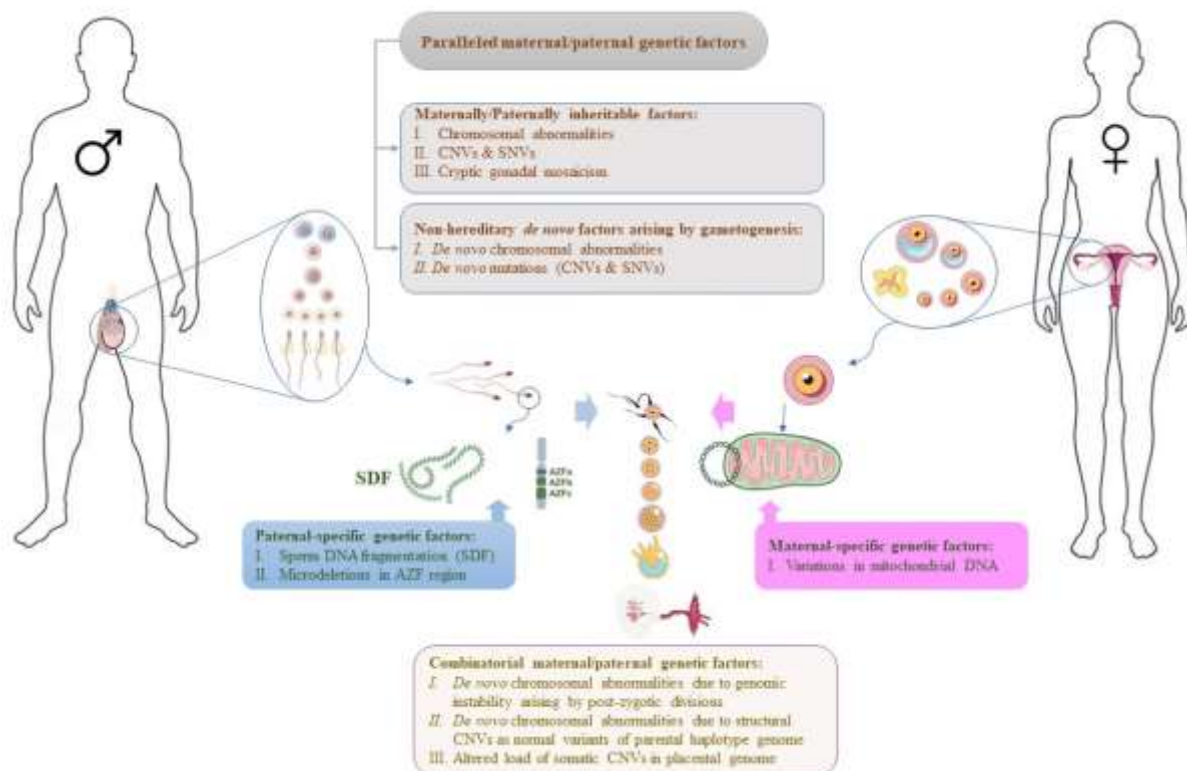
Many studies have tried to identify the genetic factors that cause RPL and have categorized them based on whether they come from the mother or father. However, there is often no distinction between specific maternal or paternal factors and genetic factors inherited in parallel from both parents. These jointly inherited genetic factors could come from two sources: 1) genetic factors derived from the maternal or paternal genome, including chromosomal abnormalities, mutations that affect cellular processes, and

detrimental alleles of common genetic variations, such as those found in thrombophilic and immunoregulatory genes; 2) genetic factors that arise during the gametogenesis process in the maternal or paternal germ layer, including chromosomal abnormalities and mutations that affect cellular processes.

**Maternally/ Paternally inheritable genetic factors**

**Chromosomal abnormalities**

Chromosomal abnormalities are a significant genetic factor that contributes to RPL. These abnormalities can be inherited from both mother and father [7] and affect the process of implantation, maintaining the embryo, and the development of the fetus. However, the impact of maternally and paternally inherited factors can vary.



**Fig. 1.** The schematic image illustrates the genetic factors involved in RPL and their genomic origins. The Figure was partly generated using Servier Medical Art, provided by Servier, licensed under a Creative Commons Attribution 3.0 unported license.

While maternal chromosomal abnormalities can affect the embryo from the beginning of fertilization and during oocyte maturation, the paternal genome activation and subsequent zygotic genome activation (ZGA) occur at the 4-8 cell stage in human embryos. Therefore, genetic abnormalities in sperm (variants of paternal origin) may not affect pre-ZGA embryonic development but may relate to defects in later embryonic development or abnormal intrauterine fetal development [8]. The rate of gross chromosomal abnormalities in either or both parents within the RPL population is approximately 2-4%, including asymptomatic individuals with balanced translocations or inversions [9, 10]. However, according to recent studies by Ahangari et al. [11] and Pal et al. [12], couples who suffer from RPL are more likely to carry chromosomal abnormalities, with a rate of 3.74% to 9.88%. The most common chromosomal abnormality in RPL couples is balanced translocation, which accounts for 38% to 47.05% of cases. Inversions account for 29.41% to 34.70% of cases, followed by numerical abnormalities (11.76% to 16.50%) and Robertsonian translocations (10.70% to 11.76%), as reported by different groups [11-13]. Interestingly, female carriers of chromosomal aberrations tend to be more frequent than male carriers among RPL couples [11, 14].

### **Submicroscopic genomic rearrangements**

Diagnostic techniques based on microarray technology have revealed new molecular insights from the analysis of submicroscopic chromosomal variations. These variations, known as DNA copy number variants (CNVs),

include deletions, duplications, and complex rearrangements of chromosomal regions. They can range in size from a few hundred base pairs (bp) to several megabytes (Mb) [5]. Research on CNVs in RPL families has provided new molecular and clinical insights in recent years. For more than ten years, the association of some CNVs, especially pericentromeric abnormalities, with RPL has been identified [15, 16]. Several studies have reported a positive correlation between the overall burden of CNVs and the risk of miscarriage [17, 18]. Furthermore, recent studies have shown an increased prevalence of large pericentromeric and sub-telomeric CNVs in the genomes of female and male partners of RPL couples [17, 19]. However, the impact of these structural genomic variants on pregnancy-related disorders may vary based on their size, type, and chromosomal location, as well as the number of genes involved and whether they affect the early pregnancy period [5].

The analysis of the underlying genes of CNVs identified in the genome of parents with RPL shows that most of these variants are related to the function of the immune system, such as allograft rejection, complement cascade, antigen binding, and antigen presentation (Table 1) [17, 18, 20]. A few recurrent CNVs have been identified as associated with RPL risk, highlighting novel genes and mechanisms possibly implicated in RPL development. For example, a 60 kb duplication encompassing the GOLPH3 and PDZD2 genes (with a regulatory role in Golgi trafficking) has been identified with a 5-fold higher prevalence among RPL females than fertile women [18].

### Single nucleotide variants

In recent years, NGS methods, particularly whole exome sequencing (WES), have helped advance our understanding of RPL. These methods have identified a range of gene variants that underlie the disease. While most studies have focused on identifying RPL-associated mutations in female partners, several studies have also examined the paternal genome, revealing the role of paternal heritable variants in the pathogenesis of RPL [8]. According to Cao et al.'s report, until 2022, fourteen studies using exome sequencing have been conducted to identify potential genes and mutations associated with RPL in affected couples. The findings of these studies have linked a total of 66 maternal and 12 paternal genes with RPL. However, only two genes - *KHDC3L* and *CCNB3* - have been repeatedly reported in more than one study, highlighting the complex nature of RPL as a multifactorial disease [8]. The identified genes are involved in different functional categories of biological processes, including coagulation and angiogenesis, extracellular matrix synthesis and degradation, regulation of immune responses, and cell divisions (meiosis and mitosis) and related processes, such as DNA replication, DNA repair, and chromatin remodeling. While findings from these studies provide new insights into the pathogenesis of RPL, functional studies are needed to determine the contribution of these genetic variants in the disease and develop novel strategies for RPL management [5, 8].

Although genome-wide studies have revealed unknown single nucleotide variants (SNVs) in RPL families, many studies have focused on the

genotyping of targeted SNVs. These studies have evaluated the known genetic variations that affect biological pathways potentially involved in RPL, such as hormonal regulation, detoxification systems, thrombosis and cardiovascular system, and, more frequently, the immune response and inflammation [4]. However, the association of SNVs with RPL has been highly variable among studies, possibly due to the clinical criteria assigned for RPL definition and genetic models used in such studies [5].

Three extensive systematic reviews have examined more than 100 genes and approximately 500 SNVs, mainly conducted on female partners of RPL families [4, 21, 22]. Interestingly, all three studies reached a relatively consistent conclusion that most nearly 500 common SNVs analyzed had no significant correlation with RPL. However, some variants were modestly correlated with the increased risk of developing RPL, including those related to thrombosis and metabolism, as well as the SNVs led to over-active immunological responses, such as those investigated in the genes *interferon-gamma (IFN- $\gamma$ )*, *interleukin (IL)-10*, *KIR2DS2*, *KIR2DS3*, *KIR2DS4*, *MBL*, and *tumour necrosis factor (TNF)- $\alpha$* . The majority of these variants, about two-thirds, have only been identified in one study. These observations may be attributed to incidental associations, the cumulative impact of other unspecified genetic factors, and environmental conditions influencing the onset of the disease [5]. Therefore, further studies are warranted to uncover the role of these factors.

The known SNVs in the two significant pathways of thrombosis and immune regulation, which have a profound impact on the process of abortion, will be further investigated in the following sections.

### **Thrombophilic factors**

Several studies have suggested that thrombophilia, a condition where the blood has an increased tendency to clot, can increase the risk of RPL in women by impairing normal placental vascular function. It's important to note that the thrombosis or occlusion of placental vessels can decrease placental perfusion, which can lead to adverse pregnancy outcomes, including RPL. It's widely accepted that thrombophilia is a common risk factor for RPL and can be observed in about 40% to 50% of RPL cases [23]. Inherited variants in some thrombophilic factors have been extensively investigated for their associations with RPL, including factor V (FV), prothrombin, and homocysteine metabolism-associated enzymes, namely methylenetetrahydrofolate reductase (MTHFR), methionine synthase (MTR), and methionine synthase reductase (MTRR) [24]. It is also has been found that both paternal and maternal mutations of thrombophilic genes, including Factor V Leiden (FVL), Factor V receptor 2 (FVR2), angiotensin I converting enzyme (ACE), and Apolipoprotein E2 (ApoE2), are related to RPL [25].

ANXA5 is an anticoagulant protein found at high levels in the human placenta. The M2 haplotype of the ANXA5 gene has been identified as a hereditary thrombophilic genetic factor linked to RPL [26]. Research has shown that carriers of the M2 haplotype in the ANXA5 gene

promoter region have more than two times higher RPL risk than the general population [27]. Besides RPL, studies have also proved the association of this haplotype to various obstetric pathologies, including gestational hypertension, preeclampsia, fetal growth restriction, preterm birth, and antiphospholipid syndrome [28]. Remarkably, the paternal ANXA5 M2 haplotype has been found to have a similar effect on the risk of RPL as the maternal mutation [29]. New findings indicate that embryos carrying the M2/ANXA5 haplotype inherited from both parents are susceptible to recurrent implantation failure (RIF) [28].

### **Immunoregulatory factors**

During pregnancy, the fetus expresses the antigens of paternal origin, making it "foreign" to the mother's immune system. This means immune tolerance between the mother and fetus is crucial for a healthy pregnancy. Most studies in this area have focused on genetic variants of maternal origin, as the mother's immune system is responsible for "rejecting" the semi-allograft fetus [8]. However, recent research has focused on the immunological role of fetal cells (originating from both the father and mother) affecting the immunoregulatory process. In particular, the human leukocyte antigens (HLA)-G has a restricted expression in the EVT and functions at the maternal-fetal interface. HLA-G, a non-classical MHC Class I antigen, is coded by a gene that generates several isoforms. These isoforms are essential in sustaining a healthy maternal-fetal interface throughout pregnancy [30]. Studies on the null allele of HLA-G isoforms proved that insufficient levels of the HLA-G proteins may negatively impact the

outcome of a pregnancy [31]. In light of previous findings indicating minimal variability in its coding regions, attention has shifted to exploring the non-coding regions of the gene (Table 1). Surprisingly, conflicting reports have emerged on the correlation between maternal HLA-G gene variants and RPL in these investigations [8]. The role of the paternal genome and inherited alleles from the father appears to have been undervalued in this scenario. Studies have shown that numerous polymorphisms in various cytokine genes, including in pro-inflammatory (IL-1 $\beta$ , IL-2, IL-17, IL-18, TNF- $\alpha$ , and IL-33) and anti-inflammatory genes (IL-10, TGF- $\beta$ 1 and IL-6), are associated with the susceptibility to RPL [8]. Moreover, several variants in genes involved in the development and function of Regulatory T cells (Tregs) cells, notably the transcription factor FOXP3, are also significantly associated with RPL susceptibility [32, 33]. Activation of naïve T cells leads to the

induction of effector T cells, which play a crucial role in regulating T cell responses and immune tolerance. Key negative regulators of this pathway, including cytotoxic T lymphocyte-associated protein 4 (CTLA-4), programmed cell death 1 (PD1), and PD-ligand 1 (PDL1), inhibit proliferation and cytokine production in T cells. Multiple variants in these genes have been associated with RPL [34-36]. Furthermore, earlier immunological evidence has shown that the complement system plays a vital role in pregnancy. Recent findings also suggest that specific genes in complement pathways, such as complement factors D (CFD) and H (CFH), are positively correlated with an increased risk of RPL [37]. Some variants related to the genes of pro-inflammatory and anti-inflammatory factors, key regulators in the induction of Treg cells and effector T cells, and complement factors are summarized in Table 1.

**Table 1.** The maternally/paternally inheritable variants involving RPL-related immunoregulatory processes.

Categories	Genes	Variants position	Mutation type	Ref.
Immunoregulatory function in lymphocytes	<i>FKBP4</i>	c.47C>A (p. A16E)	missense	[38]
Activation of pro-inflammatory caspases by inflammasomes	<i>NLRP7</i>	c.1857_1858delAC (p.Lys619Asnfs) rs2069070395	frameshift	[39]
Regulation of innate immune system	<i>NLRP10</i>	c.1015G>T (p.D339Y) rs144864261	missense	[40]
	<i>TLR3</i>	c.2384C>T(p.Ala795Val) rs373118024	missense	[41]
SNV Activation of cytokine promoters such as the IL-13 and TNF- $\alpha$	<i>NFAMI</i>	c.522C>A (p. R189Ter) rs34963472 c.498T>G/C (p.C181G/R) rs5996153	stop gained missense	[42]
Cytokine receptor on Macrophages	<i>CSF1R</i>	c.2498C>T (p.T833M) rs780804532	missense	[40]
Immunoglobulin superfamily	<i>PSG9</i>	g.43772302C>T rs3746297	splice-acceptor	[43]
HLA class I	<i>HLA-G</i>	-725 C/G/T	5' UTR	[31]

		-1573 T/C -1746 C/A		[44]
		14 bp deletion/insertion	3' UTR	[45]
Pro-inflammatory cytokines	<i>IL-1β</i>	g.4490T>C	Upstream Variant	[46]
	<i>IL-2</i>	g.4671T>G	Upstream Variant	
	<i>IL-17</i>	g.4849G>C	Upstream Variant	[47]
	<i>IL-18</i>	137 G/C		[48]
	<i>IL-33</i>	c.-11-12267G>A	Intron Variant	[49]
Anti-inflammatory cytokines	<i>IL-10</i>	g.6195A>G -819 C/T	Intron Variant	[50, 51]
	<i>IL-6</i>	g.4481G>C	Upstream Variant	
	<i>IL-22RA2</i>	c.610 C>T		
	<i>TGF-β1</i>	c.74G>C p. Arg25Pro	Missense Variant	[52]
	<i>TNF-α</i>	g.4127C>G	Upstream Variant	
g.4682G>A		Upstream Variant	[53]	
Treg cells		c.1540-18T>A	Intron Variant	
	<i>FOXP3</i>	c.-23+2877C>T	Intron Variant	[32]
		c.-22-902A>T	Intron Variant	
CD8 <sup>+</sup> CTLs	<i>CTLA-4</i>	c.49A>T p. Thr17Ser	missense	[54]
Immune inhibitory receptor/ligand on lymphocytes	<i>PDI</i>	g.4463G>A	5' UTR	[36]
	<i>PDL1</i>	Ivs2 +6371 G>A	Non-coding	[55]
	<i>CRI</i>	c.4501A>G (p.T1501A)	missense	[41]
Complement system	<i>CFH</i>	c.2325G>T (p.E775D) rs1065489	missense	
		c.1204C>T (p.H402Y) rs1061170	missense	[37]
	<i>CFD</i>	c.744C>G (p.I248M) rs2230216	missense	
CNV		8p23.1, 10q11.21-q11.22 15q11.2	macro-alteration	
	allograft rejection signaling	<i>GSTT1</i>	22q11.23	[17]
	IL-4 signaling autoimmune thyroid disease signaling	<i>CTDSPL</i>	3p22.2	microdeletions/amplifications
	<i>HLA</i>	6p21.32		

	<i>MSR1</i> 8p22	
Immunoregulatory pathways essential for immune tolerance at fetomaternal interface	<i>PDZD2</i> multicopy duplication <i>GOLPH3</i> (61.6 kb) at 5p13.3	[18]

SNV= Single nucleotide variant  
 CNV= Copy number variants  
*FKBP4*= FKBP prolyl isomerase 4  
*NLRP7*= NLR family pyrin domain containing 7  
*NLRP10*= NLR family pyrin domain containing 10  
*TLR3*= Toll like receptor 3  
*NFAM1*= NFAT activating protein with ITAM motif 1  
*CSF1R* = Colony stimulating factor 1 receptor  
*PSG9*= Pregnancy specific beta-1-glycoprotein 9  
*HLA-G* = Major histocompatibility complex, class I, G  
*IL-1β* = Interleukin-1 beta  
*IL-2*= Interleukin-2  
*IL-17*= Interleukin-17  
*IL-18*= Interleukin-18  
*IL-33*= Interleukin-33  
*IL-10*= Interleukin-10  
*IL-6*= Interleukin-6

*IL-22RA2*= Interleukin 22 receptor subunit alpha  
*TGF-β1*= Transforming growth factor beta 1  
*TNF-α* = Tumor necrosis factor alpha  
*FOXP3*= Forkhead box P3  
*CTLA-4*= Cytotoxic T-lymphocyte associated protein 4  
*PDI*= Programmed cell death 1  
*PDL1*= Programmed death-ligand 1  
*CR1*= Complement C3b/C4b receptor 1  
*CFH*= Complement factor H  
*CFD* =Complement factor D  
*GSTT1*= Glutathione S-transferase theta 1  
*CTDSPL*= CTD small phosphatase like  
*HLA*= Human leukocyte antigen  
*MSR1*= Macrophage scavenger receptor 1  
*PDZD2*= PDZ domain containing 2  
*GOLPH3*= Golgi phosphoprotein 3

**Non-hereditary *de novo* genetic factors**

Apart from inheriting identical portions of the genome from both parents, each developing embryo carries a few distinct genetic disorders, which arise during gametogenesis and postzygotic development. *De novo*-generated mutations and chromosomal abnormalities happen due to errors in DNA replication/repair mechanisms and the chromosome segregation process. The *de novo* mutations are one of the causes of genetic variation in the population, whereas *de novo* chromosomal abnormalities are the main cause of sporadic miscarriages. These errors result in variable disorders, from SNVs and indel mutations to CNVs and microsatellite instability, genomic rearrangements, and chromosomal aneuploidies [56]. The occurrence of *de novo* genetic disorders in the zygote can result in varying levels of mosaicism, determining their clinical outcome. The consequences of the disorders depend on the developmental stage and the type of cells

involved. *De novo* mutations can occur at different developmental stages, ranging from parental germline to postzygotic, embryonic, fetal, and post-natal events. Mutational processes can also occur in various cells in a tissue, including differentiating cells, stem cells, and terminally differentiated somatic cells. These factors can determine the tissue-specific distribution and burden of *de novo* disorders that can influence the potential disease they cause. Indeed, the timing of these disorders can generate variation, from homozygosity to heterozygosity, by affecting mutation load and distribution throughout the body [57].

It is widely acknowledged that parental gametogenesis, comprising oogenesis and spermatogenesis, is the primary site where errors in genome replication/repair and chromosome segregation can result in various *de novo* genetic disorders in each subsequent generation. Although it is well-known that the incidence of these disorders increases with the couple's age,

the contribution of each parent to their occurrence is different. The male germline undergoes 23 mitoses every year. This means that, for instance, a spermatogonial stem cell in a 40-year-old man has undergone over 600 mitoses. In contrast, the number of mitotic divisions remains constant during oogenesis. However, genetic disorders have been found to accumulate in oocytes over time, possibly due to the failure of the DNA repair process. Therefore, the higher rate of mitotic divisions in the paternal germline suggests that the father has a more prominent role in the occurrence of *de novo* genetic disorders in the fetus than the mother [58].

#### ***De novo* chromosomal abnormalities:**

Numerous investigations focusing on aborted fetuses have conclusively demonstrated that major chromosomal aberrations are the prevailing reason for both sporadic miscarriages (combined prevalence from 13 studies, 45%) and RPL (six studies, 39%, 29%–50%) [59, 60]. In a recent study that examined the rate of fetal chromosomal aneuploidies in POC samples, these abnormalities were reported to account for about 60% of spontaneous sporadic pregnancy losses [61]. According to a recent survey of 1000 POC samples, autosomal trisomies are the most prevalent type of chromosomal abnormalities, comprising 59.7% of cases. Polyploidies and monosomies follow, accounting for 22% and 7.5% of cases, respectively. Structural chromosome rearrangement and multiple aneuploidies are less common, representing 7% and 3.8% of cases, respectively [62]. However, when compared to sporadic spontaneous pregnancy loss, women with RPL have a lower embryonic chromosomal aneuploidy rate [63].

Most studies examining chromosomal aneuploidy in POCs have not determined the inheritance status of these abnormalities from carrier parents, so these findings cannot be considered “*de novo*” rates of chromosomal abnormalities.

While idiopathic RPL does not encompass miscarriages resulting from chromosomal abnormalities, the origin of genetically unbalanced gametes due to meiosis errors remains uncertain in many cases. In some cases, unbalanced gametes are produced due to carrier parents, particularly carriers of balanced translocations or cryptic mosaicism in the gonads that lack the phenotype. In other cases, these disorders arise *de novo* during parental gametogenesis. Although various environmental and genetic factors may play a role in *de novo* disorders, parents’ aging is still known as the main reason for this phenomenon. Indeed, as parents age, the chances of chromosomal abnormalities in their gametes increase, thus increasing the risk of miscarriage [64].

It is widely recognized that errors during mitosis and meiosis are common among all women. However, oocyte chromosomal abnormalities are caused mainly by random errors during maternal meiosis I (MI) [65]. Studies have shown that women who experience RPL and are of advanced maternal age consistently exhibit aneuploidy [66]. This may be due to *de novo* genetic disorders resulting from the missegregation of chromosomes during oocyte maturation [65]. It has been estimated that nearly 50% of oocytes are aneuploid [67], and 30% of conceptions are lost before a missed menstruation [5].

In addition to oogenesis, *de novo* genetic disorders during spermatogenesis have been identified as the reason for chromosomally abnormal embryos and the leading cause of embryonic mortality and RPL [68]. Previous studies have reported that 40% of men who demonstrated normal sperm density and motility and experienced RPL had sperm aneuploidy. Moreover, a higher proportion of sex chromosome aneuploidy has been observed in sperm samples from men with abnormal sperm density and motility compared to men with normal sperm parameters (62% vs. 45%) [69]. Recent studies further support the role of sperm aneuploidy in unexplained RPL [70-72].

#### ***De novo* mutations**

*De novo* mutations in humans are genetic modifications that have arisen within one generation. While most of the fetal genome is inherited from parents, DNMs are responsible for introducing novel genetic diversity. DNMs can take different forms, such as SNVs, insertions, deletions, and CNVs, with varying consequences [64]. Some DNMs are harmless or even beneficial, contributing to genome evolution. However, with the advent of NGS-based technologies that allow for whole-genome scanning, numerous DNMs have been identified as a major cause of malfunctioning biological systems that lead to neurodevelopmental diseases, including intellectual disability, autism, and schizophrenia [58, 73].

One of the most significant findings about DNMs is that their number increases with parental age during conception. DNMs can arise in various biological niches. An individual's first

DNMs develop during their parents' embryonic development, starting with creating primordial germ cells (PGCs) during gastrulation. In males, DNMs develop in spermatogonial cells that continuously produce sperm as they age. In females, aging of the oocytes is not accompanied by genome replication, but mutations can still occur. For some genetic diseases, the association with paternal age is stronger than with maternal age. It is believed that the cause of this association could be imprecise genome copying during the numerous cell divisions required for continuous sperm cell production in males [64].

Most NGS-based discoveries regarding DNMs have been made using parental genomes on blood samples or fetal genomes on POC samples. This makes it difficult to determine whether the DNMs originated in the germ layer of the parents or if they arose post-zygotically in embryonic cells.

#### **Maternal-specific genetic factors**

Due to the maternal inheritance of mitochondria in the fetus, mutations and variants of the mitochondrial genome are known as maternal-specific genetic factors. Studies have revealed that mitochondria play a crucial role in the differentiation and activation of immune cells, and changes in mitochondrial DNA (mtDNA) are the primary cause of developmental disorders due to their involvement in ATP production and apoptosis [74, 75]. However, little is known about the association between mtDNA changes and RPL. One study compared mitochondrial mutations in women with RPL to those of age-matched control women. The results indicated a significant increase in heteroplasmic mtDNA

diversity in the RPL group (27%) compared to the control group (19%). However, none of the detected variations in this study were previously known to be pathogenic and, therefore, unlikely to be the cause of miscarriage [76]. Another study analyzed the complete mtDNA of 100 women with RPL and tissue from 12 aborted fetuses. The results showed a significantly increased number of variants in the RPL group, particularly in the *NADH dehydrogenase* (ND) genes, which encode the complex I mitochondrial enzyme. The presence of the T4216C (*ND1* gene) variant in 9% of RPL women and several novel pathogenic mutations suggest the role of mtDNA alterations in RPL [77].

Recently, mitochondrial respiratory chain defects, especially due to genetic changes in the genes encoding complex I in mtDNA, are known to be the main cause of some immune-related diseases [74] and the basis of carcinogenesis [75]. Therefore, such genetic changes are also considered as possible causes of RPL. For example, in a direct sequencing study, mtDNA mutations and variants were examined in women with idiopathic RPL. The results identified seven variants in mitochondrial complex I genes, four of which were synonymous (*A5153G*, *C10142T*, *A14179G*, and *C14263T*). The remaining three were non-synonymous (*T4216C*, *C12063T*, and *A12662G*) changes. The case idiopathic RPL group had significantly higher proportions of these variants than those observed in the control group [78]. In conclusion, further research is needed to understand the effect and role of mitochondrial variations in the progression of RPL, which may vary among individuals and ethnic groups.

### Paternal-specific genetic factors

Certain genetic factors that contribute to RPL are inherited from the father. One such factor is the integrity of sperm DNA, which plays a vital role in fertilization and embryo development. When sperm DNA is damaged, a condition known as sperm DNA fragmentation (SDF) can lead to lower fertilization rates, terminated implantation, and miscarriages. Fertilization using damaged sperm can cause increased DNA damage in the fetal genome, leading to defects in various stages of embryogenesis and fetal development [79]. Recent studies have found that SDF has diagnostic value and suggests the possibility of paternal genetic factors in idiopathic RPL [80].

Also, Y chromosome microdeletions have long been considered a potential paternal genetic factor in idiopathic RPL. However, while some studies have linked microdeletions in a region known as azoospermia factor or Deleted in azoospermia with spermatogenic failure and male infertility [81, 82], others have found no association between Y chromosome microdeletions and RPL [83, 84]. Further research is required to explore the role of Y chromosome microdeletions as a male factor in the pathogenesis of RPL.

### Combinatorial maternal/paternal genetic factors

With the development of advanced genomic analysis tools, it has become clear that RPL should be considered a disorder caused by intrinsic factors determined by the genome of RPL partners and/or as a result of their combination as POC. Recent evidence collected from the examination of POC samples shows that about 30-50% of these samples in RPL couples have gross chromosomal abnormalities

or other structural genomic rearrangements that can describe the cause of miscarriage. The findings of recent years have shown that only 10% of these genomic abnormalities are directly inherited from parents, and 90% of them are caused by *de novo* genomic rearrangements in POC samples. Although some of these abnormalities arise during gametogenesis (oogenesis or spermatogenesis), as discussed earlier, another fraction of *de novo* genetic disorders result from postzygotic errors initiated during early embryonic development [5]. *De novo* disorders during gametogenesis do not seem to have the exact cause as postzygotic cases. The increasing age of parents is the main reason for the increasing rate of *de novo* disorders during gametogenesis [64], while postzygotic cases result from combining maternal and paternal genomes at POC [5]. It should be noted that there is usually no distinction between *de novo* disorders during gametogenesis and postzygotic types in clinical research, so most review studies discuss them together. However, it is important to recognize these disorders' different causes to understand and treat RPL better.

Extensive research has shown that a considerable portion of aborted fetuses in cases of RPL, regardless of genetic abnormalities, exhibit developmental defects that make it unlikely for them to survive beyond the first half of pregnancy, including severe disturbances of growth and morphogenesis (e.g., embryonic growth disorganization) [85]. It has also been found that the risk of congenital severe disabilities is higher for infants born in RPL

families (4.2%) than in those without a history of previous abortion (2.5%) [86]. A large study recently conducted on nearly 250,000 infants demonstrated the association between maternal history of RPL and long-term neurological morbidity and developmental and movement disorders of the offspring [87]. This is also supported by similar findings in other long-term pediatric diseases, such as respiratory morbidity of the offspring, which is possibly explained by a common immunological etiology [88]. Recent studies have proposed the hypothesis of "unfavorable genomes" as a possible consequence of combined maternal and paternal genomes on POC, leading to genomic instability in embryonic cells and a decreased chance of viable and healthy offspring for RPL couples [89]. Based on this, it has been suggested that the genomes of POCs in an RPL family carry unfavorable genomic factors that predispose them to severe developmental disorders in pregnancy [19]. Although these are currently hypotheses that require extensive studies to be confirmed, the recent evidence from examining POC samples supports this hypothesis. For example, analysis of RPL placental villus transcriptomes has exposed clear deficiencies in the vital elements that maintain the nuclear and early cellular machinery during the fast-paced proliferation and differentiation of embryonic cells. These findings confirmed the genomic instability in embryonic cells due to abnormal chromosomal segregation, disruption of DNA replication and error repair, and increased probability of somatic mutations and structural

variants, which is aligned with the “unfavorable genomes” hypothesis [90].

Cryptic mosaicism in the germ layer of the gonad has been proposed as one of the possible causes of unexplained chromosomal abnormalities in the fetus. Nevertheless, parental gonadal mosaicism should be considered when the karyotype is normal and a specific abnormality is recurrently observed [91-93]. It has been suggested that the genomic instability found in POC samples obtained from RPL families may be caused by genetic disorders that occur either during parental gametogenesis or at the postzygotic stage due to the function of the embryonic genome. Although the cause of genomic instability in many of these cases is still unknown, it is believed that some normal variants of the population carried by the maternal/paternal haplotype genome can cause genomic instability when combined in the form of the fetal genome. This is particularly true for some large structural variants, such as CNVs, particularly in the pericentromeric and subtelomeric regions [15, 19, 94]. Despite the lack of specific phenotypic effects, these CNVs can affect the correct pairing of chromosomes in meiosis and mitosis, leading to more genomic instability and miscarriage [89].

Over the last decade, evidence gathered from analyzing CNVs in POC samples, particularly in the placenta, has shown that somatic genomic rearrangements are a characteristic of the placental genome compared to other fetal tissues. It has been postulated that these somatic rearrangements may contribute to the trophoblast invasion essential for normal implantation and placental function [95, 96]. As a hallmark of

normal pregnancy in human and mouse placental genomes, somatic rearrangements primarily affect the genes responsible for cell proliferation and trophoblast invasion. Based on these findings, it has been hypothesized that the placental genomes function similarly to cancer genomes during metastatic and invasive phases, in which spontaneous structural genomic rearrangements lead to the high heterogeneity of somatic CNVs [95]. Recent evidence has shown that the load of these *de novo* CNVs in RPL samples is half the CNV load in uncomplicated first-trimester pregnancies with similar gestational age (first-trimester elective termination), mainly due to the lower number of *de novo* duplications [5, 19]. In chorionic villi samples of the RPL couples, for instance, some genes have not shown CNVs’ enrichment compared to normal pregnancies, particularly in the genes regulated by transcription factors essential in early embryonic development and regulation of fundamental cellular processes, such as E2F, ZF5, KLF4, AP2, and SREBP [90].

In addition to widespread CNVs in the placental genome, it has also been found that some pathogenic SNVs in developmentally critical genes may also predispose to recurrent cases of fetal aberrant growth. In case-control association studies conducted on the fetal genes of POC samples, the most frequently analyzed SNVs are related to immune tolerance (*HLA*- and *KIR*-genes), inflammation (*IFN* $\gamma$ , *TNF*, *TGF-B1*, *IL*-genes), blood coagulation (*MTHFR*, *F2*, *F5*, *VEGFA*), and placental development and function (*PAPPA*, *TP53*, *NOS3*, *hCGbeta* locus) [5]. Recent NGS-based studies of parent-offspring trios have shed light on the number and

distribution of these changes in human diseases. They have also identified factors that increase the likelihood of these changes occurring in offspring [5]. New mutations are the primary cause of early-onset genetic disorders such as mental retardation, autism spectrum disorder, and other developmental diseases [58]. Further studies are needed to determine how the genomic factors of RPL couples and their combination may affect the risk of future miscarriages and developmental disorders in live offspring.

### **Immunological factors involved in RPL**

Currently, research on the role of immunological factors in RPL focuses mainly on autoantibodies in the mother's blood, natural killer cells (NKC), particularly those recruited to the decidua, serum and uterine level of cytokines, classical and non-classical HLA polymorphisms, as well as the expression of HLA antigens on the embryonic trophoblast [97-99]. The role of immunological factors in RPL can arise from two sources: 1) factors generated by the mother's immune system and decidual microenvironment established by maternal genomic background, and 2) factors originating from fetal cells due to the maternal/paternal genomic background that constitutes the fetal genome. Given that pregnancy results from incessant and reciprocal interactions between the POC (embryo and subsequently fetus and fetal membranes) and the maternal uterus, it is challenging to distinguish these immunological factors and their genetic source.

The importance of these factors varies during different stages of embryonic development. For example, the blastocyst develops trophoblast by

5-6 days after fertilization and produces chorionic gonadotropins to provide a message for recognition of pregnancy and maintenance of progesterone production. Chorionic gonadotropins also affect the uterus locally to support implantation and persistence of pregnancy by modulating the immune cells, enhancing the decidualization of stromal cells, and promoting angiogenesis [100]. The uterine glands and their secretions also play a crucial biological role in implantation, uterus acceptance, blastocyst survival, and embryo growth. The function of these glands in the implantation site is regulated by the trophoblast (with fetal origin) and by decidual stromal cells and NKC in the uterus microenvironment (with maternal origin). The delicate immunological balances among these key players are critical for the maintenance and persistence of pregnancy, especially during the first trimester [101].

Furthermore, recent studies have emphasized the role of uterine mesenchymal stromal/stem cells (MSCs), particularly decidual stromal cells, to facilitate decidualization in favor of pregnancy. Cumulative pieces of evidence indicate that the deregulation of decidualization due to defective endometrial stromal cells may lead to RPL. However, the essential molecular mechanisms underlying the defect of decidualization and its related pathogenesis remain unknown [102, 103].

### **Maternal immunological factors**

Medawar first proposed mechanisms to prevent rejection of the fetus by the mother in 1953, including physical separation of maternal and

fetal tissues by the placenta, immaturity of fetal antigens responsible for graft rejection, and maternal immunological tolerance, which reflects the involvement of fetal-maternal immunological factors in maintaining pregnancy and their potential role in RPL [104]. The activity of the maternal immune system regarding immune tolerance that can contribute to the occurrence of RPL depends on the maternal genomic background. Accordingly, maternal immune tolerance is associated with fetal antigen specificity, thus, the imbalance of systemic and local immune responses under special conditions can be considered as a leading player of abortion or fetal death [105]. Some of the known pathological processes in this regard include pregnancy infections (which lead to inducing macrophages to secrete high levels of the T helper 1 (Th1) cytokine and a disruptive cytokine balance at the fetal-maternal interface), fetal antigen recognition by maternal antibodies (such as the destruction of fetal erythrocytes by Rh antigen or the destruction of platelets by HPA-1 and HPA-2 antigens) [105-107].

Diagnosing immunological causes in an aborted fetus can be challenging due to the complex mother-fetal relationship. However, maternal immune regulation disorders are considered to be the leading cause of idiopathic RPL and are divided into two categories: autoimmune abnormalities and alloimmune abnormalities. Autoimmune abnormalities are further classified into two groups: maternal autoimmune diseases and the presence of autoantibodies in maternal peripheral blood. Alloimmune abnormalities depend on the presence of fetal antigens [8]. Figure 2 shows a summary of the immunological

etiology of RPL in the form of a flowchart.

### **Autoimmune abnormalities**

Various studies have confirmed the link between autoimmune abnormalities and RPL. The immunological factors responsible for RPL can be categorized into two groups: maternal autoimmune diseases and the presence of autoantibodies in the mother's blood. Autoimmune diseases (AIDs) are chronic inflammatory conditions in which the immune system mistakenly attacks the body's tissues, causing damage. Studies have shown that people with AID, such as APS, systemic lupus erythematosus, Sjogren's syndrome, rheumatoid arthritis, systemic sclerosis, and undifferentiated connective tissue disease, have a higher risk of RPL [8]. Apart from the direct impact of maternal autoimmune diseases in causing miscarriage, it is also possible that these AIDs contribute to RPL by producing tissue-specific or non-tissue-specific autoantibodies [8]. Non-tissue-specific antibodies mainly comprise antiphospholipid antibodies (aPLs) and antinuclear antibodies (ANAs), while tissue-specific antibodies include anti-sperm and anti-ovary antibodies. The autoantibodies aPLs are categorized into classic and non-classic. Classical aPLs include lupus anticoagulant, anticardiolipin antibody, and anti  $\beta$ 2 glycoprotein I antibodies (a $\beta$ 2GP I) [108]. Non-classical aPLs, such as anti- $\beta$ 2GP I domain I antibodies, anti-prothrombin antibodies (aPT-A), and anti-phosphatidylserine/prothrombin complex antibodies (aPS/PT), have also been found to be associated with RPL [109].

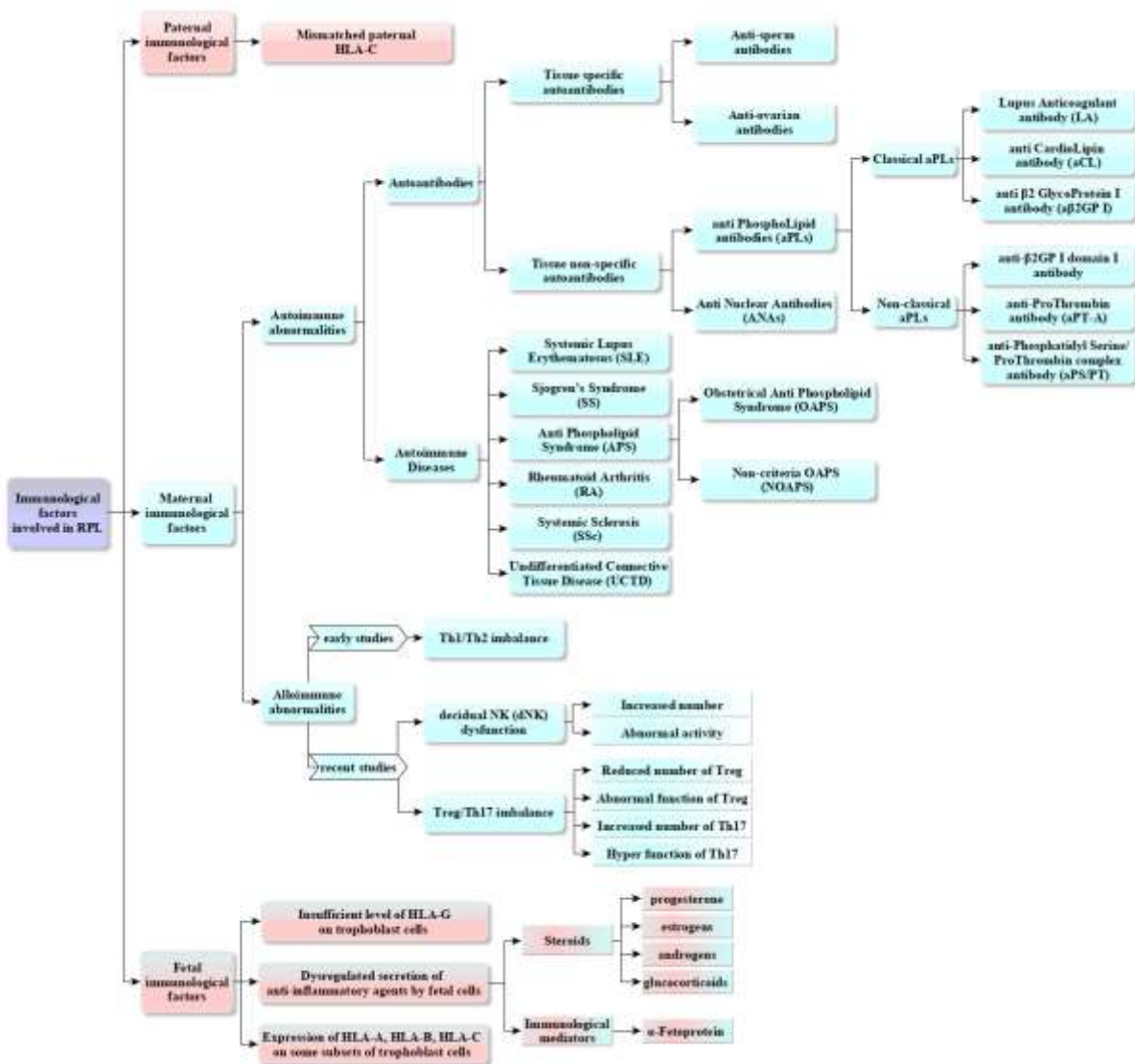


Fig. 2. The flowchart illustrates a summary of the immunological etiology of RPL.

Thrombosis, RPL, thrombocytopenia, and persistent positive serum aPLs are the key aspects of APS. Obstetrical antiphospholipid syndrome (OAPS) is the term used for APS patients with primary symptoms of pathological pregnancy, and non-criteria OAPS is used for those with typical clinical features or laboratory criteria [110].

ANAs are a group of autoantibodies that bind to DNA, RNA, protein, or molecular complexes in the nucleus [111]. More than 20 types of ANAs

have been identified [112]. Studies have found that women who experience RPL often have a higher positive rate of ANAs [111]. Overall, these autoantibodies can damage the tissues of the mother's body, the placenta, and its vascular endothelial cells, leading to pregnancy loss, in addition to their direct effect on the fetus.

**Alloimmune abnormalities**

Immune tolerance disorders in the maternal-fetal interface are often caused by alloimmune problems related to the presence of fetal antigens

in the mother's body. These problems are recognized as one of the main causes of RPL. Although environmental factors that affect the immune system's function can contribute to the occurrence of such problems, the maternal genome also plays a crucial role in the pathogenesis of the disease by determining the function of the maternal immune system [113, 114]. Alloimmune problems can influence the fate of the fetus at two-time intervals. The first is related to the uterine microenvironment before implantation, while the second is associated with the complex relationship between immune cells at the maternal-fetal interface. Both environmental and maternal genome factors are involved in both time intervals.

The successful implantation of an embryo is an important part of pregnancy maintenance. The process involves various factors, including a healthy embryo with the appropriate developmental potential from the maternal and paternal genome and a suitable intrauterine environment with appropriate hormone levels that depend on both environmental factors and the maternal genome. Before implantation, the endometrium is decidualized under the combined effect of estrogen and progesterone. The endometrium has a short window period for the blastocyst to attach when it enters the uterine cavity. During this time, endometrial stromal fibroblasts differentiate, and immune cells such as uterine NKC's and macrophages begin to accumulate [115-117]. During implantation, several interleukins (ILs) are secreted by endometrial epithelial and stromal cells, including IL-6, IL-10, IL-12, IL-15, IL-18, and leukemia inhibitory factor (LIF). These ILs form

an intricate network that orchestrates both the proliferation and maturation of uterine NKC's, controls the function of Treg and regulatory B cells, inhibiting the secretion of antifetal antibodies, and supports trophoblast invasion and decidua formation. Recent studies have shown that dysregulation of the ILs network compromises implantation and leads to RIF [118].

After successfully implanting the embryo, a complex network of cell-to-cell interactions between maternal and fetal cells is formed, creating the maternal-fetal interface. This process begins with embryonic trophoblasts differentiating into syncytiotrophoblasts and extravillous trophoblast cells (EVTs). An imbalance of immune hemostasis at the maternal-fetal interface can be a significant risk factor for RPL. The maternal aspect of this relationship is primarily related to the function of two types of decidual cells: decidual stromal cells (60%) and decidual immune cells (DICs) (40%). Uterine NKC's, which account for 70% of DICs, can recognize HLA-G on the surface of EVT's and activate themselves to secrete growth factors that promote embryonic development [119]. Additionally, macrophages (10-20% of DICs), T cells (10-20% of DICs), and dendritic cells all play a crucial role in maintaining immune homeostasis at the maternal-fetal interface [120]. However, the relationship between the number and function of these DICs and RPL is not yet fully understood.

Early studies have attributed alloimmune-related RPL to Th1/Th2 imbalance. Nevertheless, various mechanisms to prevent autoimmunity by distinguishing self-antigens from non-self-

antigens have received more attention in recent years. Peripheral tolerance to self-antigens is mainly regulated by a subset of immunosuppressive CD4<sup>+</sup> FOXP3<sup>+</sup> T cells called regulatory T cells (Tregs) [121]. Many *in vivo* and clinical studies on pregnant women have demonstrated that maternal Treg cells develop systemically and locally at the fetal-maternal interface during pregnancy, and their constant proliferation and regulation are essential for maintaining fetal tolerance. Indeed, before embryo implantation, Treg cells accumulate in the lymph nodes of the uterus in response to hormonal fluxes and stimulation by fetal antigens [122]. Therefore, Treg cells play a role in both pre-implantation and maternal-fetal interface. The imbalance between Treg and Th17 cells has increased the risk of unexplained RPL. Studies have shown that either an increase in the number and overactivity of Th17 cells or a decrease in Treg cells' number and abnormal function can significantly contribute to RPL [123]. While alloimmune problems have been the main area of study in RPL, the complexity of immune cells and factors responsible for the immune response means that current research is still in its early stages and requires more targeted investigations.

#### **Paternal immunological factors**

There have been limited studies investigating the role of paternal immunological factors in RPL due to the lack of connection between the fetus and the father's body. The research in this field has mainly focused on the immunological factors inherited from the paternal haploid genome that comprise the fetal genetic pool. For instance,

studies have indicated that the mismatched paternal HLA-C, the only classical HLA class-I expressed on the trophoblast, with the mother can lead to an alloimmune response. A study conducted to examine the frequency of paternal alleles of HLA-C\*07 and HLA-C\*17 (the most immunogenic HLA-C antigens) and the presence of HLA-specific antibodies in maternal blood revealed that HLA-C incompatibility between couples is significantly associated with unexplained RPL [124].

Recently, new findings have emerged, indicating the role of the father's immune system health status as a result of pregnancy. This discovery has drawn attention to the role of paternal immunological factors in the occurrence of RPL. For instance, a large multicentre retrospective study (iFAME-Fertility) conducted in a Dutch population showed a significantly higher miscarriage rate in men with inflammatory arthritis [125]. These findings highlight the importance of investigating the relationship between unexplained RPL and paternal immunologic factors, including immunomodulatory factors in paternal peripheral blood and seminal fluid. Therefore, a hospital-based multicentre case-control study and prospective cohort study (REMI III project) have been designed to examine this relationship [126].

#### **Fetal immunological factors**

The role of fetal cells in maintaining a pregnancy can be considered in two aspects: 1) A functional aspect that has been suggested for long time ago by Medawar's strategies is the antigen-immaturity of fetal cells that enables them to escape from recognition by maternal immune system and is mostly related to the hierarchical

levels of embryonic and fetal cells; 2) the endocrine and paracrine secretions of small molecules of nucleic acid, proteins, growth factors and anti-inflammatory agents from fetal cells that are mainly related to the maternal-paternal genomic background.

In the first trimester, the placenta forms an EVT structure that interacts with the mother's immune system. This interaction can create a microenvironment that its imbalance causes immunological attacks against the fetus, leading to miscarriage [119]. Despite such close contact, *in vitro* studies have shown any antigenic stimulation of maternal lymphocytes by fetal trophoblastic cells [127]. The best explanation for this observation is low-expressed classical HLA in trophoblast. Despite the presence of paternal HLA alleles in the fetal genome, their expression on fetal cells and their possible role in miscarriage remains controversial. Hypothetically, this is where other genetic factors could affect the outcome of HLA involvements. For example, maternal antibodies against paternal HLA antigens have been observed in 10% to 30% of expected pregnancies. However, there is no documented abortion resulting from these antibodies since new HLA antigens (from paternal sources) expressed on fetal membranes are often tolerogenic rather than immunogenic [128].

Trophoblast cells selectively express specific non-polymorphic HLA subsets, such as HLA-G, which help protect the fetus against uterine NK activity [119]. However, some studies have been published on natural fertility and pregnancy in individuals who lack both HLA-G alleles, which is found in different ethnicities with a prevalence of 0.6% to 11% [129]. On the other hand, it has

been found that some subsets of trophoblast cells may slightly express classical HLA antigens such as HLA-A, HLA-B, or HLA-C. Consequently, their presence might be a possible cause for abortion due to the development of antibodies with high specificity to HLA antigens [130].

Another suggested approach fetal cells use to maintain pregnancy is their immunosuppressive properties. It has been revealed that fetal and placental cells can release anti-inflammatory agents as endocrine (transferred from the fetal to the maternal bloodstream) and paracrine (locally in the site of the fetal-maternal interaction) manner, leading to suppression/modulation of the maternal immune system [106]. Generally, these compounds can be divided into two groups: 1) steroids, including progesterone, estrogens, androgens, and glucocorticoids that enter the maternal bloodstream and originate from fetoplacental, adrenal, and fetal liver tissues and play a role in immunological processes related to pregnancy, from implantation to delivery [131]; 2) another group is complex protein molecules that transfer most of the immunological information from the fetus to the mother, such as  $\alpha$ -Fetoprotein that is produced in the fetal liver and transmits the immunosuppressive message to the mother [132].

Moreover, *in vitro* studies have shown that fetal cells have more potential for immunosuppression than their adult counterparts. For example, studies using co-culture systems have shown that unlike adult dendritic cells, fetal ones strongly reduce the proliferation of CD8<sup>+</sup> T cells. Additionally, these fetal cells induce Treg cells

and inhibit TNF- $\alpha$  production in T cells, thereby preventing pro-inflammatory responses [133]. The transcriptomic investigation of adult and fetal dendritic cells revealed more than three thousand differentially expressed genes [133]. Another example is MSCs, whose notable feature is immunosuppression [134]. Numerous clinical trials have been conducted on this feature and its application in cell therapy for inflammatory diseases [135, 136]. Accordingly, many studies comparing MSCs of fetal sources with adult ones have disclosed more immunosuppressive activity of fetal tissue-derived MSCs [137, 138].

## Conclusion

Based on the literature, nearly 50% of RPL cases are considered idiopathic miscarriages, and immunological factors have been suggested as one of its possible causes. The genomic similarity of aborted fetuses during RPL is the same as sibling similarity in a family, so identifying a common factor that causes RPL in a mother is very challenging. Therefore, the

immunological factors involved in miscarriage can either result from the maternal genomic background or fetal origin due to the maternal-paternal genomic background. Considering that pregnancy results from the constant interaction between the pregnancy product (placenta, fetus, and fetal membranes) and the mother's uterus, the precise determination of immunological factors and their genomic origin would be promising to develop new therapeutic approaches to help couples.

## Ethical Considerations

All ethical considerations were followed in compiling this work.

## Funding

This study was supported by Yazd Reproductive Sciences Institute.

## Conflict of Interest

The authors declare that there is no conflict of interest regarding this article's publication and financial issues.

## Acknowledgements

The authors acknowledge Dr. Nasrin Ghasemi for her support as the head of the Abortion Research Center.

## Authors' Contributions

All the authors have played an equal role in implementing this project.

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