Review

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Understanding recurrent pregnancy loss: recent advances on its etiology, clinical diagnosis, and management

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Abstract: Recurrent pregnancy loss (RPL) has become an important reproductive health issue worldwide. RPL affects about 2%–3% of reproductive-aged women, and makes serious threats to women's physical and mental health. However, the etiology of approximately 50% of RPL cases remains unknown (unexplained RPL), which poses a big challenge for clinical management of these patients. RPL has been widely regarded as a complex disease where its etiology has been attributed to numerous factors. Heretofore, various risk factors for RPL have been identified, such as maternal ages, genetic factors, anatomical structural abnormalities, endocrine dysfunction, prethrombotic state, immunological factors, and infection. More importantly, development and applications of next generation sequencing technology have significantly expanded opportunities to discover chromosomal aberrations and single gene variants responsible for RPL, which provides new insight into its pathogenic mechanisms. Furthermore, based upon patients' diagnostic evaluation and etiologic diagnosis, specific therapeutic recommendations have been established. This review will highlight current understanding and recent advances on RPL, with a special focus on the immunological and genetic etiologies, clinical diagnosis and therapeutic management.

Keywords: etiologic diagnosis; genetic etiology; next generation sequencing; recurrent pregnancy loss; therapeutic recommendations.

Introduction

Recurrent pregnancy loss (RPL) is defined as two or more spontaneous pregnancy losses before 20 weeks of gestation, according to the guidelines from the European Society of Human Reproduction and Embryology (ESHRE) and the American Society for Reproductive Medicine (ASRM) [1]. The latest Chinese expert consensus also suggested that RPL refers to experiencing two or more consecutive pregnancy losses with the same spouse before 28 weeks of gestation in China [2]. With regard to the terminology of RPL, ESHRE recommends "recurrent pregnancy loss" as a general term to describe repeated pregnancy losses and use of "recurrent miscarriage" to describe recurrent intrauterine miscarriages [3]. Furthermore, the definition of RPL encompasses pregnancy losses after spontaneous conception and assisted reproductive treatment (ART), but excludes pregnancy failures such as ectopic, molar pregnancies and implantation failures [4]. Whether RPL includes non-consecutive losses or biochemical losses remain controversial among countries or international societies. Likewise, estimating the prevalence of RPL is challenging, because it is influenced by distinct patient populations, different guidelines and varied RPL definition. For instance, a previous report showed that recurrent miscarriage (RM) has a prevalence of about 0.8%–1.4% if only repeated intrauterine pregnancy losses (confirmed by

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ultrasonography and histopathology) are counted. Nevertheless, if non-visualized pregnancy losses, such as biochemical pregnancy loss, are added, the prevalence rises to 2%–3% [5].

RPL is an important reproductive health issue worldwide, and, of note, it is regarded to be one of the most challenging aspects in the field of reproductive medicine, owing to the fact that more than 50% of couples with RPL have no clear etiological explanation [6]. Unlike sporadic pregnancy loss, RPL needs more medical intervention and appropriate pregnancy monitoring during future pregnancies. Nowadays, it is widely accepted that RPL belongs to a multifactorial disease, and its causation has been attributed to various factors including chromosome aneuploidy, anatomical uterine defects, immunological dysfunction, endocrinological abnormalities, genetic variants as well as lifestyle influences [7]. However, URPL, where these aforementioned factors were devoid of causative influences, brings tough challenges in both diagnosis and treatment for clinicians and also gives rise to a severe psychological distress on the couples.

Here, we aim to give an in-depth review of current understanding of the diagnosis, pathophysiology and management of RPL. In particular, novel insights into immunological and genetic etiologies of RPL will also be outlined.

Epidemiology of RPL

According to previous epidemiological surveys, the incidence of RPL has increased annually worldwide [1]. Advanced maternal age is one of the main risk factors for RPL, and numerous studies have shown that the risk of miscarriage is lowest in women between the ages of 20 and 29, rises significantly after the age 30, and can grow more than 50% above the age 45 [8]. RPL is a unique reproductive problem with the following characteristics: (1) the risk of RPL is directly related to previous pregnancy outcomes [9–12]. Patients who have a history of miscarriage only once are less likely to have another. In addition, the risk of RM increases by 10% for each additional miscarriage, especially in patients who have 3 or more miscarriages, where the risk of RM can exceed 40% [8]. (2) The incidence of clinically identified RPL (1%) is higher than the expected incidence (0.34%) [13]. (3) Compared to spontaneous miscarriage, the fetal chromosomal abnormalities in RPL couples are less frequent [14]. (4) RPL tends to affect women with specific reproductive characteristics, such as a history of intrauterine growth retardation (IUGR), late miscarriage, stillbirth or neonatal death, ectopic pregnancy, or preterm birth [15–18].

Studies have shown that several RPL can be attributed to adverse environmental conditions, psychological factors, and poor lifestyle habits [19]. The adverse factors, including exposure to air and water pollution, radiation, unsuitable environmental temperature and malnutrition, have been considered as prominent risk factors for RPL [20]. Also, mental conditions like distress, hostility and anxiety of pregnant women were strongly associated with RPL [2, 21, 22]. In addition, many domestic and international guidelines or consensus have highlighted the correlation between adverse lifestyle habits and psychological factors and RPL [3, 19], suggesting that RPL patients should quit smoking, avoid alcohol abuse, maintain an appropriate body mass index (BMI), and get necessary psychological counseling.

Primary risk factors for RPL

The etiology of RPL is complicated and multiple risk factors, mainly including genetic factors, anatomical structural abnormalities, endocrine dysfunction, prethrombotic state, immunological imbalance and infection, have been identified as primary causes of RPL. Among these factors, genetic factors and maternal immune dysregulation have been regarded as two of the most important causes of RPL, and we will discuss in great detail in the following sections. Anatomical structural factors, including congenital uterine anomalies (septate uterus, arcuate uterus, bicornuate uterus, unicornis uterus, and double uterus), and acquired uterine structural abnormalities (intrauterine adhesion [IUA], hysteromyoma, and adenomyosis), accounted for approximately 16% of RPL, which is significantly higher than incidence of uterine anatomical abnormalities in the general female population [23, 24]. Frequently, women with endocrine disorders, such as abnormal thyroid function, hyperprolactinemia (HPRL), luteal phase deficiency (LPD), and polycystic ovary syndrome (PCOS), predispose them to RPL, and account for approximately 8%–12% of RPL patients [25]. Studies have shown that endocrine related pregnancy failures are likely to occur early in pregnancy, indicating that hormonal regulation might play crucial roles in attachment and early implantation of an embryo into the uterus [26]. The maternal hypercoagulable state of blood is a physiological change in the blood system during normal pregnancy [27], and when excessive hypercoagulability of the blood occurs, the blood develops a pathological condition which predisposes to thrombus formation called prethrombotic state (PTS). Many studies have demonstrated that PTS, either hereditary or acquired, is associated with an increased risk of RPL [28, 29]. Hereditary PTS is caused by mutations in genes related to anti-coagulation, coagulation and fibrinolysis, and acquired PTS mainly involves antiphospholipid syndrome

(APS), acquired HHcy, as well as other thrombogenic disorders. Hereditary PTS due to FVL and coagulation Factor II mutations are rare in the Han population, while the deficiency of Protein C and Protein S are more common, which is reversed in Europeans and Americans.

Bacterial and viral infections may be also related to increased risks of RPL. The association of bacterial vaginosis and dysbiosis of the reproductive tract with RPL has been reported but the results are inconsistent [30, 31]. The incidence of chronic endometritis, possibly induced by infection, is significantly higher in patients with infertility, recurrent embryo implantation failure (RIF) and RPL, and oral antibiotic therapy may improve the pregnancy prognosis in this group of patients [32]. However, more randomized controlled trials (RCT) are necessary to confirm and to provide more reliable evidence for verifying association of infection with the reproductive failures.

Immunological factors in the etiology of RPL

Maternal immune dysregulation is associated with RPL

Maternal immune dysregulation has been regarded as one of the leading causes in the etiology of RPL [33]. Most notably, patients with a history of RPL may often have autoimmune abnormalities or alloimmune problems [20]. It is likely that autoimmune abnormalities contribute to RPL by producing tissue-specific or tissue non-specific autoantibodies. The tissue non-specific antibodies mainly comprise antiphospholipid antibodies (aPLs) and antinuclear antibodies (ANAs), etc. And the tissue-specific antibodies comprise anti-sperm antibodies and antiovarian antibodies, etc. Moreover, these autoantibodies could attack the mother's own tissues and placenta, and damage the vascular endothelial cells, thus leading to pregnancy loss. Alloimmune problems which associated with disruptions of maternal-fetal interface immune tolerance, were also a major cause of RPL. Although immune related factors have become the focus of current research into the development of RPL, the immune cells and factors responsible for the immune response are intricate, and the current research is still in its infancy, and more in-depth and specific studies need to be conducted in the future.

Autoimmune diseases and RPL

Autoimmune diseases (AID) are chronic inflammatory diseases in which the immune system responds to self-antigens and causes damage to its own tissues. Many studies have shown that AID such as APS [34], systemic lupus erythematosus (SLE), Sjogren's syndrome (SS), rheumatoid arthritis (RA), systemic sclerosis (SSc) and undifferentiated connective tissue disease (UCTD) have a higher risk of RPL. Women with SLE, for instance, have a high rate of spontaneous miscarriage (approximately 20%), especially in middle and late stages of pregnancy, and the stillbirth rate is 2–4 times higher than that of normal pregnant women. Importantly, many common autoantibodies have been found in these co-morbid AID patients like aPLs and ANAs, which were also related to RPL. aPLs, sensitive indicators of intrauterine distress or death, are found in almost all stillborn patients with SLE. Classical aPLs include lupus anticoagulant (LA), anticardiolipin antibody (aCL) and anti β2 glycoprotein I antibodies (aβ2GP I) [35]. Currently, some non-classical aPLs, such as anti-β2GP I domain I antibody, anti-prothrombin antibody (aPT-A), and anti-phosphatidylserine/prothrombin complex antibody (aPS/PT), have also been found to be associated with RPL [36]. ANAs belong to a class of self-antibodies that bind to DNA, RNA, protein or molecular complexes in the nucleus, and more than 20 types of ANAs have been identified at present. Studies have found that women with more times of pregnancy loss are often associated with higher positive rate of ANAs. Furthermore, positive rate of ANAs in RPL patients is 8%–50%, 38.1% in patients with two consecutive miscarriages and increases up to 43.5% in those with three or more consecutive miscarriages.

Antiphospholipid syndrome and RPL

APS is also an AID, which is characterized by recurrent arteriovenous thrombosis, RPL, thrombocytopenia, and persistent positive serum aPLs. In addition, APS whose main clinical characteristic with pathological pregnancy is called obstetrical antiphospholipid syndrome (OAPS) [37], and APS with only typical clinical manifestations or only typical laboratory diagnostic criteria are called non-criteria OAPS (NOAPS). aPLs, whose main target antigens are β2GP I and prothrombin, play crucial roles in the pathogenesis of RPL caused by APS. Studies have shown that under pathological conditions, more open form of β2GP I, which prefers to bind to aPLs, accumulates on the decidual endothelial cells as well as villi and extravillous trophoblasts. The aPLs and β2GP I complex can then activate complement system, which in turn dampens the coagulation system by increasing fibrin deposition and placental vascular thrombosis. Moreover, this complex not only affects angiogenesis and spiral arteries remodeling, but

also inhibits proliferation and differentiation of trophoblast cells and induces apoptosis by promoting an inflammatory response (Figure 1). Importantly, it is worth mentioning that RPL patients caused by OAPS needs more attention and non-standard aPLs tests are worth using in patients negative for classical aPLs but with significant clinical symptoms.

Alloimmune risk factors in RPL

Furthermore, early studies have attributed alloimmune related RPL to Th1/Th2 imbalance, and in recent years, an increasing number of studies have found that Treg/Th17 imbalance is associated with a higher risk of URPL. Treg has a strong immunosuppressive activity by decreasing inflammation, and in contrast, Th17 promote the pro-inflammatory responses [38]. IL-17, which is produced by Th17 cells and plays an important role in the acute inflammatory response and autoimmune response, has been identified as a prominent immunological risk factor for RPL. Also, studies have shown that either increase in the amount and hyperfunction of Th17 cells, or abnormal function and reduced number of Treg cells, could greatly contribute to RPL. Furthermore, abnormal number and activity of NK cells are also reported to be involved in the pathogenesis of RPL. Decidual NK (dNK) cells have capacities to modulate trophoblast invasion, and decidua and spiral arteries remodeling through producing various of cytokines and growth factors. The disturbance of these processes caused by dNK cells dysfunction may be related to RPL [39]. Furthermore, the detection of the number and function of circulating peripheral blood NK cells and uterine NK cells is useful in diagnosis and treatment of RPL.

Immune homeostasis at fetal-maternal interface links to reproductive success

Pregnancy begins with successful implantation of an embryo, and a successful pregnancy requires a combination of factors,

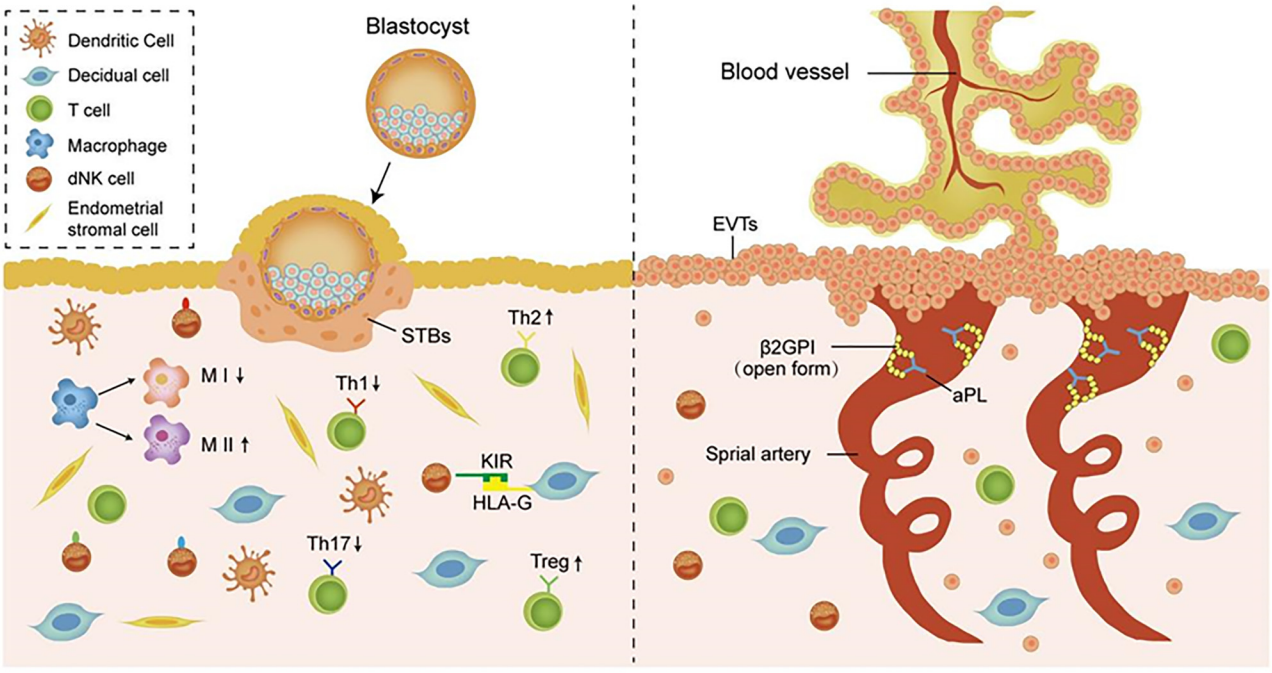

Figure 1: Dysregulation of immune and coagulation systems at the maternal-fetal interface in RPL patients. Decidual lymphocytes modulate immune response via interacting with each other and also regulate invaded trophoblasts and decidual cells by secreting various cytokines and chemokines. Notably, disturbance of immune regulation at the interface, which leads to impaired immune tolerance, is frequently associated with RPL. dNK cells are the dominant immune cells at the interface, and they induce immune tolerance by recognizing different kinds of HLA molecules on EVTs by encoding various types of inhibitory KIR. Recent single-cell transcriptomics analyses show that reduction of specific dNK subsets such as $CD27^{-}CD11b^{-}$, pregnancy trained dNK, and $CSF1^{+}$ $CD59^{+}$ $KIRs^{-}$may be related to RPL. Furthermore, increased Th1/Th2 and M1/M2 ratios, reduced immunosuppressive Treg cells and DC, and increased pro-inflammatory Th17 cells, have also been identified as risk factors for RPL. In addition, aPLs interacts with open form of β2GP I in endothelial and placental cells, and can activate the complement system, which inhibits angiogenesis and the development of spiral arteries, as well as proliferation and differentiation of trophoblast cells. RPL, recurrent pregnancy loss; EVTs, extravillous trophoblast cells; KIR, killer cell immunoglobulin like receptors; DC, dendritic cells.

in general, including a healthy embryo with a great growth potential and an excellent intrauterine environment with appropriate hormone levels [20]. The endometrium undergoes decidualization under the combined action of estrogen and progesterone before embryo implantation. After the blastocyst enters the uterine cavity, the endometrium has a short window period for the blastocyst to attach. During the window period, the endometrial stromal fibroblasts spontaneously differentiate into metaphase cells and the immune cells, such as uterine NK cells and macrophages, begin to accumulate. Multiple risk factors, such as chronic inflammation of the endometrium, luteal insufficiency, metabolic (obesity) and endocrine disorders (abnormal thyroid function), and luteal insufficiency can lead to impairment of the decidualization process, which is in turn linked to RPL [21, 40, 41].

Once the embryo is implanted, the maternal-fetal interface is formed, and constitutes a complicated cell-to-cell interaction network from cells of maternal and fetal origins (Figure 1). At the interface, the fetal trophoblasts differentiate into syncytiotrophoblasts (STBs) and extravillous trophoblast cells (EVTs). The maternal decidual cells contain decidual stromal cells (DSCs) and decidual immune cells (DICs). DICs account for approximately 40% of all cells at the maternal-fetal interface in early pregnancy and are intricately regulated during the implantation process to ensure maternal tolerance of fetal placenta. Immune imbalance at the maternal-fetal interface is also an important risk factor for RPL. As an example, uterine NK cells which comprise 70% of DIC can recognize HLA-G on the surface of EVTs and activate uterine NK themselves to secrete growth factors that promote embryonic development [39, 42]. However, relationship between the number and function of uterine NK cells and RPL has not been fully clarified. Macrophages (10%–20%), T cells (10%–20%) and dendritic cells (DCs) also play important roles in maintaining immune homeostasis of the maternal-fetal interface [43–45]. Remarkably, the critical roles of Treg cells in pregnancy have been demonstrated in multiple recent studies [46], and the immunosuppressive tolerogenic DCs can induce Treg cell differentiation in the endometrium. Nevertheless, there is a significant reduction in tolerogenic DCs in RPL patients, emphasizing the importance of tolerogenic DCs in maternal-fetal immune regulation [47].

More importantly, several single-cell transcriptomics analyses of decidua have been recently performed, which offer more detailed information on the regulation of immune responses at the maternal-fetal interface. These studies not only revealed new subsets of uterine immune cells, but also uncover their potential contributions to RPL. For instance, Vento-Tormo et al. showed that three major subsets of dNK cells were detected in the first-trimester decidua, among which $CD27^{-}CD11b^{-}$ dNK subset was the dominant cell population that has the typical dNK functions [48]. And Wang et al. investigated abnormal properties of dNK subsets in decidua from RPL patients and found that $CD27^{-}CD11b^{-}$ dNK1 cells show a strikingly lower frequency, implying its causal roles in RPL [49]. Furthermore, a significant decrease of pregnancy trained dNK (PT dNK), which shows the comparable function of $CD27^{-}CD11b^{-}$ dNK1 subset, has been demonstrated to be associated with RPL [50, 51]. A new subset of dNK cells ($CSF1^{+}$ $CD59^{+}$ $KIRs^{-}$) was also revealed in decidua, and their reduction might be linked to URPL [49]. Besides, new subsets of dDCs, T cells, macrophages, decidualized stromal cells and the cell-cell interaction were also investigated, and these novel findings indeed expand our knowledge of the maternal-fetal interface immune tolerance as well as its relation to the onset of RPL [52–54].

Maternal and paternal genetic factors account for RPL

Maternally derived numerical and structural chromosomal abnormalities

Embryonic chromosomal aneuploidies have been regarded as the leading genetic cause of human pregnancy loss, and account for approximately 60% of sporadic spontaneous pregnancy losses [55]. Of these cases, autosomal trisomies (59.7%) have been identified as the most common chromosomal abnormalities, followed by polyploidies (22%), monosomies (7.5%), structural chromosome rearrangement (7%) and multiple aneuploidies (3.8%) [56], according to a recent survey of 1000 products of conception (POC) samples. These chromosome abnormalities mostly originate from random errors in maternal meiosis I (MI) that possibly result from abnormal segregation during oocyte development, and advanced maternal age is generally associated with higher rates of embryonic aneuploidies during pregnancy [57]. Many studies have shown that embryonic chromosome anomalies can also explain RPL [56, 58]. However, compared with sporadic spontaneous pregnancy loss, women with RPL have a decreased embryonic chromosomal aneuploidy rate [59]. Several recent studies have estimated that carriers of structural and numerical chromosomal abnormalities are detected in about 3.74%–9.88% of RPL

couples [60, 61]. Out of these chromosomal abnormalities detected in RPL couples, balanced translocation (38.00%–47.05%) is the most frequent chromosomal abnormality, followed by inversions (29.41%–34.70%), numerical abnormalities (11.76%–16.50%), and Robertsonian translocations (10.70%–11.76%) [60–62]. Interestingly, several studies have shown that among RPL couples with chromosomal aberrations, female carriers tend to be more frequent than male carriers [60, 63]. As well, studies have demonstrated that RPL women of advanced maternal ages have consistently shown a higher incidence of aneuploidy [64, 65].

Maternal aneuploidy and chromosomal mosaicism

Maternal mosaicism may also contribute to embryonic chromosomal aneuploidy, in addition to random errors in the first meiotic division of the oocyte and parental carrier of chromosomal aneuploidy [66]. Chromosomal mosaicism is commonly known as the existence of genetically different cells within an individual, and mosaicism occurs during embryonic development as a result of chromosomal segregation in the course of mitotic cell division. Robinson et al. have reported that eight RPL couples with normal karyotypes show a recurrence of the same chromosomal abnormalities (trisomy 15, 16, 22, and triploidy), possibly arising from maternal mosaicism [67]. To date, many groups have reported that chromosomal aneuploidies were detected in the POCs in several cases with a history of RPL and normal karyotype of fibroblasts or lymphocyte genome. Their results revealed that maternal mosaicism most probably gives rise to embryonic chromosome aneuploidies [68–71]. Recently, Ghevaria et al. [72] has focused on the maternal aneuploidy mosaicism (germinal mosaicism) that arises either in the primordial germ cells (gonadal mosaicism) or during the premeiotic mitotic divisions of the oogonia. Their study estimated that the incidence of premeiotic aneuploidy in non-selected oocytes was greater than 10%. In fact, the recurrence of numerical anomalies is rare, and maternal mosaicism must be taken into account when the parental karyotype is obviously normal and the same anomaly has been observed repeatedly.

Maternal factors associated with genome stability of early embryonic cells

With respect to RPL, the origins of embryonic chromosomal abnormalities can be explained by parental carriers and maternal mosaicism, as well as maternal age. Indeed, other causes, which are independent of these risk factors and could contribute to embryonic or fatal aneuploidy, still need to be explored in RPL population. For example, Burada et al. [73] reported a couple with three distinct consecutive trisomic pregnancies, in whom the existence of abnormal karyotypes and mosaicisms in both members was excluded. Delhanty et al. [74] have shown that some patients are inclined to repeatedly produce abnormal embryos with mosaic aneuploidy in *in vitro* fertilization (IVF) cycles (chaotically dividing embryos). These observations bring about the possibility that variations in certain factors of maternal origin in the oocytes may disturb the division of embryonic cells, independent of maternal ages. Zhang et al. have discovered two heterozygous deletions in *KHDC3L* gene (p.E150_V160del and p. E150_V172del) in two women with a RPL history, and their findings further demonstrated that deficiencies of *KHDC3L* may be associated with genomic abnormalities and apoptosis of early embryonic cells [75]. Significantly, McCoy et al. [76] first reported that common variants in *PLK4* gene, involved in the regulation of centriole duplication during mitosis, are linked to an increased risk of aneuploidy during human early embryo development. Women with high-risk genotypes produce fewer embryos, possibly due to a lower potential for development of early embryos. Zhang et al. [77] investigated association between maternal variant (rs2305957) of *PLK4* gene and blastocyst formation rate in a Chinese cohort, and found that infertile females with A/A genotype presented a lower rate of blastocyst formation compared to those with genotype of either A/G or G/G.

Maternal inherited thrombophilic factors variants

A series of studies suggest that thrombophilia might increase women's risk for RPL by impairing normal placental vascular function. It is important to note that thrombosis or occlusion of placental vessels may decrease placental perfusion, which may contribute to adverse pregnancy outcomes including RPL [78]. Notably, several inherited variants in three thrombophilic factors, such as factor V gene, homocysteine metabolism associated enzymes (*MTHFR*, *MTR* and *MTRR*), and prothrombin gene (*PTG*), have been extensively investigated for their associations with RPL [79, 80]. *FVL*, a missense mutation in the factor V gene (p.R506Q), is the most extensively studied thrombophilic variants in patients with RPL. The allele frequency of *FVL* varies among different populations, with the highest in Europe (4.4%) and the

lowest in Asia (0.6%) [81]. *FVL* can induce activated protein C (APC) resistance, which in turn leads to a hypercoagulable state [82]. However, the susceptibility of *FVL* to RPL is controversial, and both strong and negative associations with RPL have been reported [83–85]. Recently, Eslami et al. [86] have performed a large scale meta-analysis which includes 10,410 RPL cases and 9,406 controls, and their results indicated that *FLV* presents a significant association with RPL in overall population analysis, and the positive association was also observed in Asian, European, Africa populations but not in south Americans. In addition, the prothrombin G20210A mutation, giving rise to elevated mRNA and protein expression of prothrombin, is associated with an increased risks of thrombosis [87]. Liu et al. also reported a systematic review and meta-analysis of 89 studies containing 30,254 individuals, and their finding revealed that women carrying the *FVL* mutation or the G20210A mutation had a higher risk of developing RPL [88].

Homocysteine (Hcy), a sulfur-containing amino acid, is generated during the metabolism of methionine. Three main enzymes, including methylenetetrahydrofolate reductase (*MTHFR*), methionine synthase (*MTR*), and methionine synthase reductase (*MTRR*), are involved in this metabolic process. Hyperhomocysteinemia has also been regarded as an important risk factor for thrombophilia [89]. Moreover, multiple common genetic variants in these enzymatic genes have been demonstrated to be associated with increased plasma homocysteine concentrations [90], and the maternal accumulated homocysteine could further damage the endothelium and impair placental function, which may be related to placenta-associated pregnancy complications, such as RPL, placental abruption and preeclampsia [91]. Among these variants, *MTHFR* C677T and A1298C which contribute to decreased enzyme activities, have been extensively investigated. Interestingly, earlier studies have shown that maternal homozygous carriers of *MTHFR* C677T, under the condition of folate deficiency, can cause elevated levels of Hcy [92]. However, the contribution of maternal *MTHFR* C677T and A1298C to RPL risk is controversial [93–96], and inconsistent associations of *MTHFR* variants with RPL were also observed among racial and ethnic groups [97]. Only a few studies have been performed to evaluate the genetic association of variants in *MTR* and *MTRR* genes with RPL. Sata et al. [98] have found that women with the *MTR* c.2756 A/G genotype show a decreased risk of RPL in Japanese population. Kim et al. [99] also reported that female carriers of the *MTR* c.2756 A/A have a higher risk of RPL in Korean population. Furthermore, Zhang et al. [100] have shown that heterozygous women for *MTRR* c.66 A > G show a higher RPL risk in Chinese population. However, the susceptibilities of *MTR* c.2756 A > G and *MTRR* c.66 A > G to RPL were not replicated in Vietnamese population [80].

Maternal factors implicated in modulating immune responses

The fetus expresses the antigens of paternal origin, and just like a semi-allograft, the fetus is foreign to the maternal immune system [101]. Therefore, fetomaternal immune tolerance is essential for the maintenance of a healthy pregnancy. Studies have paid more attention to investigate the RPL-related immunological risk factors, such as autoantibodies, peripheral and uterine NK-cells, and regulatory T cells [102]. Indeed, several genetic variants of maternal origin, affecting immunoregulatory process, have been identified as risk factors for RPL. Specially, there is considerable interest in the *HLA-G* (a non-classical MHC Class I antigen), which shows a restricted expression in the EVT and functions at the maternal-fetal interface. Previous studies indicated that a low degree of polymorphism has been observed in the coding regions of *HLA-G* gene. Therefore, many studies have focused on variants in the non-coding regions, such as 5′ upstream promoter region (–725 C/G/T [103], –1573 T/C and –1746 C/A [104]) and 3′ untranslated region (14 bp deletion/insertion [105]) of *HLA-G* gene [103, 106, 107]. However, both positive and negative association of maternal *HLA-G* gene variants with RPL were reported by these researches. Meuleman et al. systematically investigated the association of maternal *HLA* alleles, including *HLA I*, *HLA II* and non-classical *HLA I* alleles, with RM, and their meta-analysis indeed demonstrated that specific maternal *HLA* alleles were associated with RM susceptibility. However, they considered that there was no consistent conclusion for the associations of *HLA* and RM, due to the existing information and selection bias [108]. More strict inclusion criteria, including ethnicity, diagnosis, and sample size, are required to reveal the association of *HLA* alleles with risks for RPL. Furthermore, studies have demonstrated that multiple interleukin (*IL*) genes are associated with RPL susceptibility. Among those, the pro-inflammatory *IL* gene variants, including *IL-1β* (–511 T/C), *IL2* (–330 A/C), *IL-17* (rs2275913), *IL18* (137 G/C) and *IL33* (rs16924159), have been shown to be risk factors for RPL, because these factors stimulate the activity of the immune response [109–112]. Besides, anti-inflammatory *IL* genes, such as *IL-10* (2195

A/G and −819 C/T) and *IL-6* (−634 C/G), contribute significantly to an increased risk of RPL [113–116]. A rare mutation (c.610 C>T) in *IL22RA2* has been detected in 4 out of 328 Chinese women with a history of RPL, and in silico analyses show its potential pathogenic roles in RPL. In addition to *IL* genes, other cytokines, such as *TGF-β1* (rs1800471) [117] and *TNF-α* (−863 C/A and −308 G/A) [118, 119], were also linked to RPL risks. Moreover, the transcription factor Forkhead Box P3 (*FOXP3*), which plays an important role in the development of Treg cells, is significantly linked to RPL susceptibility [120, 121], that is also supported by a recent large-scale meta-analysis. Cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) functions as a negative regulator of T-cell activation and participates in regulating the T cell response and immune tolerance. Several studies have revealed an association of multiple genetic variants of *CTLA4* with RPL [116, 122–124]. It is well known that programmed cell death 1 (PD1) and programmed cell death ligand 1 (PDL1) signaling has been demonstrated to be a key negative regulator of T cell activity by inhibiting proliferation and cytokine production [125]. Indeed, Hayashi et al. [126] have found that two variants (rs36084323 and rs3481962) of *PD1* gene confer a significant risk for RPL. Cho et al. [127] have demonstrated the positive relationship between *complement factor D* (*CFD*) and *complement factor H* (*CFH*) and RPL risks, suggesting the important roles of the complement system in gestation.

Whole exome sequencing in women with RPL

Next generation sequencing (NGS), particularly the exome sequencing, has been extensively used to identify the gene variants underlying human genetic diseases. To date, fourteen studies have been reported to perform exome sequencing for identification of potential genes and genetic mutations contributing to RPL (Table S1 and S2 [128–141]). Most of these studies focus on the discovery of RPL-associated mutations in women patients, and a total of 66 candidate maternal genes and 12 paternal genes have been reported to be associated with RPL (Table 1). Intriguingly, among these genes, only two genes (*KHDC3L* and *CCNB3*) were repeatedly detected in two different studies (Table S1), suggesting heterogeneous and complicated conditions of RPL. Moreover, these studies also show that these RPL-related genes are mainly enriched in biological processes, including coagulation and angiogenesis, extracellular matrix (ECM) composition and degradation, and immune regulation (Table 1). Although these findings provide novel insights into the pathogenesis of RPL, functional

Table 1: RPL associated genes revealed by whole exome sequencing analysis

Groups	Biological processes	Maternal genes	Paternal genes
1	Coagulation and angiogenesis	*ANXA5, F13A1, F5, FGA, FN1, THBS1, THBD, FLT1, ADAMTS1*	*KDR, ITGB1*
2	Chromatin remodeling	*BPTF*	*BPTF*
3	Cell surface receptor	*FGFR2, APP, EPS15, MS4A14*	
4	DNA methylation	*DNMT1, MECP2, MBD4*	*MECP2*
5	DNA replication, RNA transcription and splicing	*CBX3, NCOA1*	*EFTUD2, POLR2B*
6	DNA repair	*REXO4, MSH2*	
7	Extracellular matrix composition and degradation	*COL6A3,TNC, MMP10, MMP9, LAMA2*	*MMP2, LAMA4, COL4A2*
8	Immune regulation	*FKBP4, TLR3, NLRP7, NLRP10, CR1, NFAM1, CSF1R, PSG9*	–
9	Mitosis	*PLK1, CENPH, PIF1, CEP250, CCDC68*	*PLK1, BUB1B*
10	Meiosis	*CCNB3*	
11	Membrane transporters	*ABCA4, ABCB5, TCN2, CAPS, SLC2A7*	–
12	Membrane protein that mediates cell adhesion	*TRO, CDH11, CDH1, YES1*	–
13	Metabolic process	*FOXA2, IDO2, OSBPL5, GFA*	
14	Oocyte maturation and early embryonic development	*KHDC3L, PADI6*	–
15	Transcription factors	*BNC2, EPAS1, SOX21*	*SOX21*
16	Others	*AMN, BMP7, TRAF3IP1, LIFR, DNAH11, PLCD4, IFT122, AL078585.1*	–

The name of genes are given in italics. RPL, recurrent pregnancy loss.

studies are required to delineate the contribution of these genetic variants to RPL, thereby accelerating the development of specific treatments for RPL.

Paternal chromosome abnormalities

In addition to genetic abnormalities in females, more and more investigations indicated that male factors, especially genetic causes, also make a large contribution to RPL [142]. Owing to the fact that paternal genome activation and subsequent zygotic genome activation (ZGA) occurs at the 4–8 cells stage in human embryos [143], genetic abnormalities in sperm (variants of paternal origin) may not impact pre-ZGA embryonic development, but may relate to defects in later stages of embryonic development or abnormal intrauterine fetal development.

Most importantly, numerical or structural chromosome abnormalities in spermatozoa have been identified as the leading causes related to embryonic mortality and RPL. In general, these abnormalities might arise *de novo* during spermatogenesis [144], and in turn, give rise to chromosomally abnormal embryos. Ramasamy et al. [145] reported that sperm aneuploidy was detected in 40% of men who exhibited normal sperm density and motility and experienced RPL. Intriguingly, compared to men with normal sperm parameters, a higher proportion of sperm sex chromosome aneuploidy was observed in men with abnormal sperm density and motility (62% vs. 45%). And, abnormal strict morphology (based on Kruger strict criteria) was greatly associated with increased rates of sperm aneuploidy. Likewise, a recent systematic review [146] shows that the incidence of sperm aneuploidy was significantly higher in men whose spouses have a history of RPL, which is mostly in agreement with previous studies [147–149]. A recent multicenter study also supports the notion that the sperm aneuploidy may contribute to unexplained RPL [150]. In addition to the sperm *de novo* chromosome abnormalities, carriers with chromosomal aberrations were also detected in couples with RPL or RM. Several earlier studies [151, 152] showed that 2.7%–6.5% of RPL couples carried structural chromosome rearrangement, which was significantly higher than that reported in general human population (about 0.5%) [153]. Recently, Li et al. performed a comprehensive survey among 3,235 RPL couples, and revealed that 121 individuals (3.74%), including 75 women and 46 men, harbor structural or numerical chromosome abnormalities [60]. Among these variants, balanced translocations were identified as the dominant abnormalities (46/101), following by inversions (42/101), numerical abnormalities (20/101), and Robertsonian translocations (13/101). Dong et al. [154] investigated chromosome abnormalities in 1090 couples with RM through low-pass genome sequencing approach, and 127 chromosomal abnormalities were uncovered in 126 couples. Similarly, a higher prevalence of balanced translocations was observed among these RM-affected couples (61.9%, 78/126). Balanced translocation commonly has no effect on the carriers' phenotype but the carriers can generate genetically unbalanced gametes due to the meiotic errors.

Sperm DNA fragmentation

Sperm DNA integrity is essential to fertilization and embryo development, and sperm DNA damaging, also called sperm DNA fragmentation (SDF), can result in low fertilization rates, embryonic arrest, and pregnancy loss [155]. Indeed, fertilization by the damaged spermatozoon may lead to an increased DNA damage in embryo genome, which could result in defects at different stages of embryogenesis and fetal development [156]. Robinson et al. [157] completed a meta-analysis of 16 cohort studies involving 2969 couples, and their findings showed that high sperm DNA damage was strongly associated with increased risk of sporadic miscarriage. Also, according to a meta-analysis of 19 studies involving 1182 couples with unexplained RM and 1231 couples without RM, Dai et al. demonstrated that increased levels of SDF are significantly associated with unexplained RM, suggesting that the male factor SDF may be implicated in the pathogenesis of RM [158]. Furthermore, several large-scale meta-analyses have been recently performed to investigate the correlation between SDF and RPL. McQueen et al. enrolled 579 men with a history of RPL and 434 male controls, and their results showed that SDF is clearly associated with RPL [159]. To assess the relationship between SDF and idiopathic RPL, Tan et al. conducted a meta-analysis of 12 prospective and 2 retrospective studies including 530 men with a history of RPL and 639 fertile controls, their findings indicated that SDF provides a diagnostic value over standard semen analysis, and also raises the possibility that SDF could serve as a paternally derived genetic origin of idiopathic RPL [160].

Y chromosome microdeletions

Reijo et al. [161] first reported in 1995 the finding of Y chromosome microdeletions, that was identified in 12 men with azoospermia. Deletion in this region, also described as azoospermia factor (AZF) or Deleted in

AZoospermia (DAZ), is associated with spermatogenic failure and male infertility. Further analysis showed that AZF locus can be divided into three subregions (AZFa, AZFb and AZFc), and microdeletions in these subregions can lead to varying degrees of spermatogenetic failure. Intriguingly, microdeletions in the overlapping region between AZFb and AZFc may relate to the range of sperm counts (no measurable sperm to normal sperm count) [162]. Thus, several studies have investigated the relationship between Y-chromosome microdeletions and RPL. Dewan et al. [162] first demonstrated that men from RPL couples present a significantly higher prevalence of Y-chromosome microdeletions in the proximal AZFc region than men from fertile or infertile couples. This finding was mostly supported by two subsequent studies [163, 164]. However, Dai et al. [165] recently evaluated the contribution of Y chromosome microdeletions to RPL in Northeast China, by recruiting 1,072 men with a history of RPL and 971 infertile and 200 fertile males as controls. Their finding did not reveal an association between Y chromosome microdeletions and RPL, which is in accordance with previous studies [166–168] in Sri Lanka, Mexican and Iran. Till now, many possible explanations, such as selection of sequence-tagged sites (STSs) marker [165], extraction of DNA from different cells (peripheral blood or spermatozoa) [168], and sample size [163], have been suggested for this obvious discrepancy. Importantly, further investigations and more information are needed to explore the roles of Y chromosome microdeletions, as a male factor, in RPL pathogenesis.

Single gene variants of paternal origin

Association of maternal *MTHFR* C677T polymorphism with a susceptibility to RPL has been well investigated. Several recent studies have also found that paternal C677T polymorphism is connected with RPL aetiology [93, 169]. It is now widely accepted that thrombophilia is a common risk factor for RPL and can be observed in about 40%–50% of RPL cases [78]. Ozdemir et al. found that both paternal and maternal mutations of thrombophilic genes, including *FVL*, *FVR2*, *ACE*, and *ApoE2*, are related to RPL [170]. ANXA5, an anticoagulant protein that is abundantly expressed in human placenta, has also been identified as a RPL associated factor. Bogdanova et al. [171] first verified that carriers of M2 haplotype in the *ANXA5* promoter region have over 2-fold higher risk of RPL compared to general population, which is indeed supported by a recent large-scale meta-analysis [172]. So far, the association of *ANXA5* M2 haplotype with RPL has been replicated in multiple populations, such as Japanese [173], Malay [174], and Indian [175], but not in Estonia and Denmark. Remarkably, Rogenhofer et al. [176] demonstrated that, in line with maternal mutation, paternal *ANXA5* M2 haplotype also confer an increased risk of RPL. Ubiquitin pathway proved to be implicated in fertilization and embryo development, and *USP26* (ubiquitin specific peptidase 26) gene variants have been previously identified as risk factors for male infertility. Moreover, according to the findings from Asadpor et al. [177], a *USP26* haplotype, comprised of 370–371 insACA, 1423 C > T and 494 T > C mutations, shows significantly higher frequency in men with a history of idiopathic RPL than that of in fertile men, supporting the important roles of paternal genetic variants of *USP26* gene in RPL etiology.

Diagnosis and therapeutic management of RPL

Guidance on diagnosis and management of RPL

At present, therapeutic recommendations for RPL are mainly to take some symptomatic treatments based upon the underlying causes [20]. Many guidelines have been formulated and developed by many domestic and international societies, such as ESHRE, Royal College of Obstetricians and Gynaecologists (RCOG) [178], ASRM [1], German Society of Gynecology and Obstetrics (DGGG), Austrian Society of Gynecology and Obstetrics (OEGGG) and Swiss Society of Gynecology and Obstetrics (SGGG) [179]. Although the definitions and diagnosis of RPL are not completely uniform across national guidelines, the management of this condition is similar. Systematic etiologic screening is recommended for patients with RPL. However, in patients with a history of only one miscarriage, systematic etiologic screening is not recommended except for a clear family history or clinical manifestations of associated diseases. Depending on the underlying causes of RPL, corresponding managements or therapeutic intervention are established (Table 2, and more details are listed in Table S3).

Novel strategies for management of RPL

Nowadays, in addition to routine management for RPL patients with clear cause, a number of novel trail treatments have emerged. For instances, several studies have shown that multivitamin supplement especially Vitamin D was effective for RPL patients, but the supplement doses

Table 2: The recommended managements of women with recurrent pregnancy loss based on etiology

Etiology	Clinical testing	Therapeutic managements and intervention	References
Genetic factors	Karyotype analysis of RPL couples and aborted embryo tissues	Genetic counseling before the next pregnancy PGT	[3]
Anatomical structural factors	Routine pelvic ultrasonography	Monitoring during pregnancy Hysteroscopic mediastinectomy is used for severe IUA Hysteroscopic myomectomy is used for submucosal fibroids Prophylactic cervical cerclage is recommended for cervical insufficiency	[3, 30]
Endocrine factors	Endocrine testing	Thyroxine management to keep the level of TSH controlled in an appropriate range. Exercises, oral hypoglycemic drugs and insulin injection for abnormal glucose metabolism Progesterone supplementation for LPD	[3, 55–57]
Prethrombotic state	Hereditary PTS screening and classical detection of aPLs excludes APS-related acquired PTS	Monotherapy or combination therapy of LMWH and LDA	[1, 58–61]
OAPS	Classical aPLs testing including LA, aCL, and β2-GPIAb	LDA combined with LMWH, plus HCQ or glucocorticoids if necessary	[3, 58–61]
NOAPS	Individualized testing for nontypical aPLs profile	The managements were not unified. Existing treatments can still refer to the treatment of OAPS and need to be administered by experienced obstetricians and rheumatologists.	[3, 58–61]
Immune factors	ANAs and ENAs profile screening	Fertility counseling immunosuppressants, combined with LDA or LMWH if necessary	[3]
Infection factors	Infection-related factors checking during pregnancy	Appropriate antibiotic treatment for patients with obvious symptoms of genital tract infection.	[3]
Other factors	Recording clinical information of RPL couples	Keep good lifestyle habits and stay away from adverse environments	[3]

LDA, Low-dose aspirin; LMWH, low-molecular weight heparin; HCQ, hydroxychloroquine; IUA, intrauterine adhesion; LPD, luteal phase deficiency; OAPS, obstetrical antiphospholipid syndrome; NOAPS, non-criteria obstetrical antiphospholipid syndrome; RPL, recurrent pregnancy loss; PTS, prethrombotic state; aPLs, antiphospholipid antibodies; ANAs, antinuclear antibodies; LA, lupus anticoagulant; aCL, anticardiolipin antibody.

are inconsistent among these studies [180–182]. Additionally, immunotherapy for RPL has been intensively studied, and many different treatment options have been developed. Typically, treatments such as lymphocyte immunotherapy (LIT), anti-tumor necrosis factor (TNF) alpha agents, granulocyte colony stimulating factor (G-CSF) and paternal cell immunization all have been applied to clinical treatment [183–185]. However, several studies indicated that immunotherapy treatments like intravenous immunoglobulin G (IVIG) cannot increase live birth rates of RPL [3, 183]. Some meta-analysis and RCT trails also showed that immunomodulatory agent may benefit on certain subgroup of RPL patients [186–189]. Therefore, the latest consensus concluded that immunotherapy treatments are recommended to use only in clinical research. Furthermore, therapeutic intralipid infusion and endometrial scratching have also been evaluated, but the results indicated that these treatments were not effective for RPL [2, 46].

Interestingly, many groups have investigated the mesenchymal stem cells (MSCs) based therapy in abortion-prone mouse model, and demonstrated that MSC treatment could improve pregnancy outcome through modulating the immune responses at the maternal-fetal interface [190]. Eskandarian et al. showed that MSC therapy could remarkably reduce the abortion rate of abortion-prone mice, possibly by increasing the frequency of uterine DCs (uDCs) [191]. Kahmini et al. injected adipose-derived MSCs into the abortion-prone mice, and their results indicated that MSCs can improve the tolerogenic microenvironment

at the maternal-fetal interface by decreasing the infiltration of $CD49b^+$ NK cells to the decidua [192]. Farrokhi et al. revealed that MSCs based therapy could also reduce the abortion rate of the abortion-prone mice through enhancing Tregs expansion as well as upregulating expression of Treg-related genes [193]. Furthermore, Xiang et al. found that injection of exosomes from MSCs into uterine horns could modulates T cell and macrophages responses at the maternal-fetal interface, thereby leading to a decreased abortion rate of abortion prone mice [190]. Indeed, these attempts and explorations open up new opportunities for the development of novel therapeutic options to improve pregnancy outcome of RPL patients.

Recent advances in treatment of unexplained RPL

URPL is a diagnosis of exclusion and should first meet the diagnostic criteria for RPL, while autoimmune diseases, PTS, anatomical abnormalities of the reproductive system, endocrine and chromosomal as well as genomic abnormalities should be excluded. Several studies suggest that unexplained RPL may have a genetic predisposition [194] and may be also related to some unknown immune factors. Several meta-analyses have been conducted and revealed multiple susceptible genes showing association with idiopathic or unexplained RPL, indicating that genetic factors may play important roles in the etiology of URPL. Interestingly, most of these genes were enriched in important biological processes, such as immune response, coagulation, metabolism, angiogenesis [195, 196] and placental function. In addition to genetic causes, many evidences also suggested that immune related factors may explain the etiology of URPL. van der Zwan et al. found that the altered proportion of the small activated subset of dDC cells and $CD8^+$ T cells that exhibited cytotoxic properties may contribute to URPL [44]. Svensson-Arvelund et al. also demonstrated that a high ratio of M1/M2 ratio of macrophage may associate with pathogenesis of URPL [43].

Due to lack of sufficient evidences, clinical management of URPL is challenging and limited. On one hand, routine use of (IVIG) [197–199], liposomes, LIT, anticoagulant therapy (LDA or LMWH), glucocorticoids, cyclic Cyclosporine A (CsA), G-CSF, TNF-α inhibitors and other treatment methods are not recommended [2]. On the other hand, it is recommended to encourage unexplained RPL patients to participate in clinical research programs according to their specific conditions. Importantly, decoding the genetics that drive URPL may provide a promising avenue to develop new treatments for this disorder.

Conclusions and outlook

The present review has summarized the current knowledge and recent advances on the etiology, diagnosis and treatment strategies of RPL. These recent updates in the understanding of RPL would provide novel insights into the origin of pregnancy loss, and may further facilitate the development of novel strategies for management of women with RPL.

Although significant progress has been made in this field, more than half of the RPL patients have no clear explanation for their failed pregnancy. Currently, there is a lack of effective treatment options for these URPL patients, owing largely to limited understanding of the mechanisms underlying URPL. The supportive care for couples with unexplained RPL is primarily recommended by many international guidelines [200]. To improve pregnancy outcomes of URPL patients, future studies should pay more attention to these patients. Importantly, the newly developed technologies may provide opportunities to uncover the patient-specific pathogenic mechanisms. For example, single-cell RNA sequencing of patient-specific pregnancy-related tissues, and investigation of patient-specific endometrial [201] and placental organoids [202], are promising options for discovering causal factors in specific URPL couples. Furthermore, these patient-specific findings may lead to development of personalized therapeutic strategies.

A large number of genetic predisposing factors to RPL have been identified currently (Figure 2). As aforementioned, the RPL-associated genes are enriched in various functional categories, such as chromosomal abnormalities, thrombosis and immune responses. Studies have shown that discoveries of genetic variations in RSA population, especially the unexplained recurrent spontaneous abortion, should contribute to explaining the unknown causes of RPL [196, 203]. Furthermore, the interplay between genetic and environmental factors indeed plays an important role in understanding pathogenesis of many human diseases [204]. However, till now, fewer large-scale studies have been conducted to investigate the complex interactions between RPL susceptible genes and environmental factors. Typically, the interaction of *MTHFR* gene variants with folic acid supplements has been extensively investigated, and this gene-nutrient interplay plays critical roles in multiple pregnancy complications [205]. Future work, focusing on the combined effects of environmental risk factors and genetic variants, is expected to yield more results to delineate the etiology of RPL.

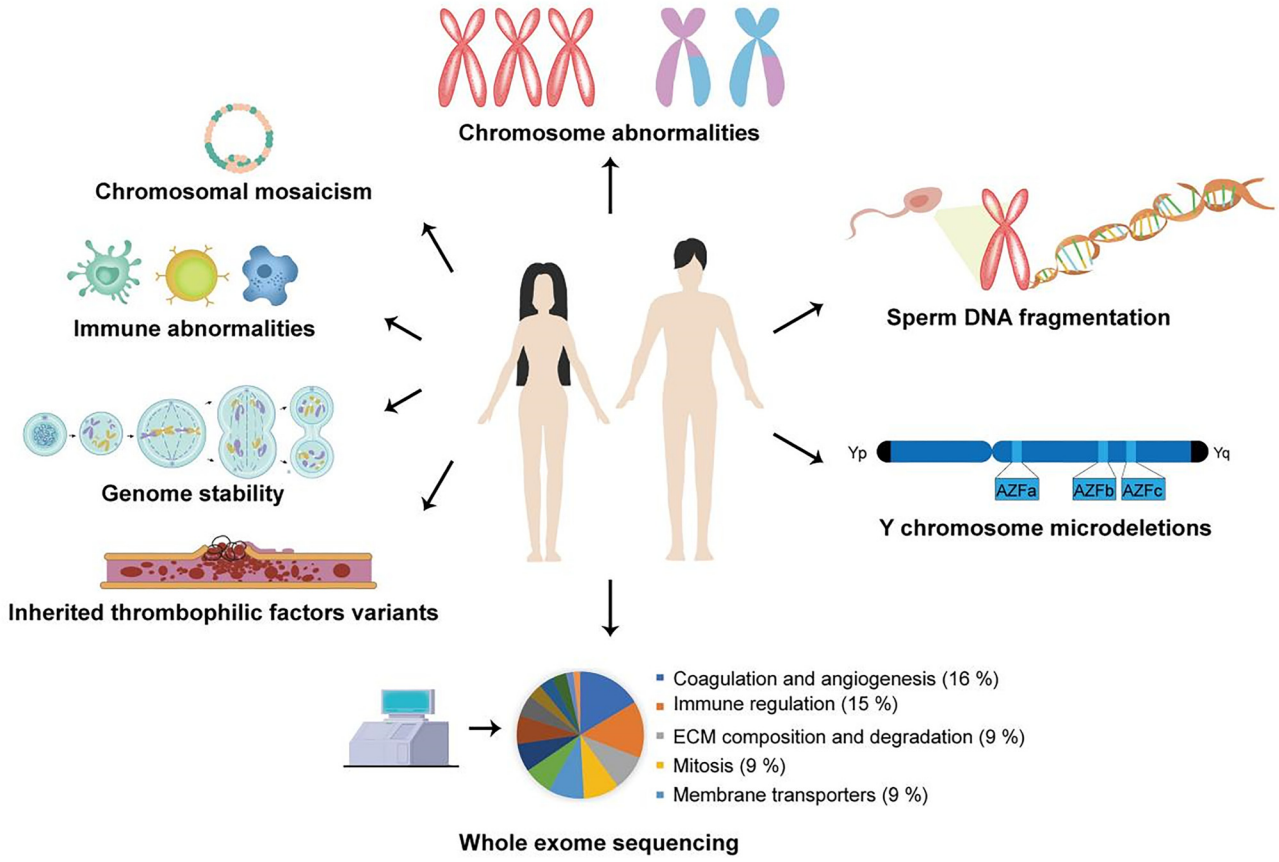

Figure 2: The genetic factors of maternal and paternal origins contribute to RPL. Studies have identified many RPL associated genetic factors, including maternally and paternally derived chromosomal abnormalities, maternal aneuploidy mosaicism, sperm DNA fragmentation, Y chromosome microdeletions and variants of functional genes involved in coagulation, immune response, ECM remodeling, and so on. ECM, extracellular matrix; RPL, recurrent pregnancy loss.

NGS, such as whole exome sequencing [206, 207], has been widely used for discovering genetic causes of human inherited disorders, including the RPL. Until now, more than 14 studies have been performed to identify RPL causing mutations (Table 1). Indeed, a series of disease associated rare variants have been detected, and these newly identified genes provide novel insights into the pathogenesis of RPL as well as explanations for a specific subset of RPL patients. Nonetheless, the causal roles of most variants remain to be disclosed, and further functional studies, based on cellular and animal models, could be undertook to investigate functional relevance of these genetic variants. In addition, NGS is also a powerful tool in clinical diagnosis [208], and for instance, it shows advantages over conventional cytogenetic analysis. Dong et al. demonstrated that compared to conventional karyotype analysis, low-pass genome sequencing significantly enhanced diagnostic yield of chromosomal abnormalities [154] in RPL population.

In conclusion, both clinical investigations and fundamental researches are required to accelerate our understanding of RPL and to develop new strategies to improve outcomes of these complex medical conditions.

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