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Genetic Regulation of Recurrent Spontaneous Abortion in Humans

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Recurrent pregnancy loss, defined as a pregnancy failure occurring before 24 weeks of gestation more than two or three times according to most definitions, is a fertility defect encountered in 1-5% of the patients. This defect is of course of multifactorial origin. Among the possible origins of recurrent pregnancy loss are uterine structural defaults, defective ploidy control of the embryo, defective immunological dialog between the embryo (or the fetus) and the uterus sometimes in relation with immunological disorders (such as autoimmune diseases), thrombophilia, and free radical metabolism imbalance. Numerous studies attempted to correlate variants of genes supposed to be intervening in the different facets of the early maternal–fetal or maternal–embryonic dialog, and eventually modify the outcome of fertilization, leading to success or failure of post-implantation development. The objective of the present review is to portray the major genes and gene polymorphisms studied for their putative association with recurrent pregnancy loss. Most of these genes have been studied as candidate genes for which strong biological arguments were put forward as to their putative involvement in recurrent pregnancy loss. They were mostly studied by genetic analysis, often in various populations of different ethnic origins, throughout the world. Some of these studies were available only in English as abstracts and were nevertheless used if the information was given with enough detail. With the space being too short to depict all the available literature, different major pathways relevant to the scientific question are presented without any attempt to hide the fact that discordant views often aroused for a given gene. *(Biomed J 2014;37:??-??)*

Key words: genetics, infertility, recurrent miscarriage

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In humans, pregnancy may be interrupted at various stages, leading to abortion, and this process occurs repetitively in some patients. Repetitive interruptions of pregnancy are variously referred to as recurrent spontaneous abortion (RSA), recurrent pregnancy loss (RPL), recurrent miscarriage (RM), idiopathic recurrent miscarriage (iRM), or unexplained recurrent spontaneous abortion (URSA). These different terms indicate terminations of pregnancy occurring more than 2 times before 24 weeks of gestation, in most definitions of the syndrome. In humans, it appears that at least 15% of the recognized pregnancies end in miscarriage. In an estimated half of the cases, chromosomal anomalies such as aneuploidies are observed.[1]

In this review, we will attempt to summarize current knowledge on the genetic basis of recurrent abortions, and will not deal with environmental or stochastic factors that can lead to isolated abortive events. Clearly, for instance, the increase of chromosomal anomalies leading to aneuploidy and mosaicism in blastocyst[2] is generally associated with mother's age (even though paternal age may also be considered as a risk factor)[3] and is beyond the scope of the present review. However, genetic defects leading to a quasi-systematic increase of aneuploidy will be considered. Other possible genetic causes of recurrent abortion may be found in developmental defects of the blastocyst or the embryo that do not involve gross chromosome anomalies, but probably much more frequently involve a defective materno-fetal immunological dialog, or a defective uterine function impeding early development. There is a plethora of articles dealing with genetic polymorphisms associated with RSA. However, until now, the genetic polymorphism analysis has been concentrated on a limited number of genes. We will not attempt to review exhaustively the existing literature, which analyzes various populations worldwide, but

rather attempt to categorize the factors that were analyzed according to their biological functions and summarize their putative effects.

Candidate gene approaches

Feto-placental side

Genetic bases of defective chromosome content or structure

In a biological sense, genetic defects leading to abortion may originate from two partners: The conceptus and the uterus. From the conceptus side, it is well-established that chromosomal anomalies lead to implantation defects. However, they occur mostly stochastically; to be the genetic cause of a recurrent loss, a genetic defect leading to systematic chromosome anomalies has to be found, as nicely reviewed recently by Kurahashi and coworkers.[4] Abnormal chromosome structure may originate from defects in the checkpoint mechanisms that constitute a quality control factory of the cell, which is able to detect meiosis anomalies and is called Spindle Assembly Checkpoint (SAC), constituted by proteins such as Mad1, Mad2, Bub1, Bub3, BubR1, and Mps1.[5] A very recent study addressed this question on the general issue of defective reproduction performance, by screening two candidate genes, aurora kinase B (*AURKB*) and synaptonemal complex protein 3 (*SYCP3*). In the *AURKB* gene, the authors identified low-frequency (0.5%) non-synonymous variants (c. 155C.T, c. 236T.C, and c. 880G.A) inducing the changes p.A52V, p.I79T, and p.A294T in the polypeptidic chain, respectively. 236T >C and 880G >A were associated with antecedents of pregnancy losses.[6] This preliminary study is one of those that pave the way toward evaluating genes playing a role in the quality of the meiotic progression. Two other genes have been investigated to this respect in miscarriage, *BUB1* and *MAD2*.[7] The authors showed firstly that as generally assumed, half the embryos from spontaneous miscarriages have an abnormal karyotype. By western blotting, the authors quantified the two proteins and estimated that they were under-expressed in pathological cases. The authors also isolated chorionic villi where they targeted *BUB1* and *MAD2* expression by short hairpin RNA (shRNA) and showed that this induces an abnormal karyotype at a ~ sixfold increased frequency, compared to controls (indicating that mitotic defects can also be induced when these genes are dysfunctional). The role of *MAD2* in such defects was independently confirmed by another study on trisomic abortuses.[8] Clearly, therefore, anomalies in spindle assembly can lead to aneuploidies, and infertilities in the most severe cases, such as in the case of the recently identified mutation of the cohesin *STAG3*, leading to premature ovarian failure,[9] but less severe mutations in

the *SAC* genes could lead to variants that have more subtle effects. There is clearly space for studying this aspect of spindle stability on a wider basis than what has been performed up to now in the context of recurrent miscarriage.

Placental developmental defects

The mammalian placenta plays several crucial roles in the initiation and maintenance of pregnancy. In humans, it constitutes the main immunological interface between mother and child, allowing tolerance between two genetically distinct organisms. It is the major exchange surface between the two; at the end of pregnancy, 600 ml of maternal blood circulates through the placenta at each minute. Finally, it secretes important hormones such as the human chorionic gonadotrophin (hCG) and the human placental lactogen (hPL) that have immunotrophic functions. In humans, the functional unit of the placenta is the placental villus, a densely branched tree (up to 16 ramification levels), covered by a syncytium, the syncytiotrophoblast.[10] Normally, there is an intimate relation between the endometrium and the invasive human placenta; therefore, it can be suspected that at least in some cases, defective invasion can be caused by genetic specificities of the placenta and may lead to abortion. In a recent study of Lyu and coworkers,[11] the authors investigated the placental villi in the cases of idiopathic miscarriages, comparing four controls and four miscarriages with expression microarrays, after collecting the villi at 10-12 weeks. In this study, the authors uncovered essentially modifications in the management of oxidative stress, and more specifically, in glutathione metabolism. Glutathione depletion leads to accumulation of superoxide radicals that play a toxic role in the cells;[12] indeed, glutathione peroxidases are the only enzymes able to manage H_2O_2 in the mitochondrial context (since catalase is not expressed in the mitochondria), and require reduced glutathione (GSH) to convert H_2O_2 into water.[13] As well, genes involved in mitochondrial function, especially oxidative phosphorylation, appeared affected. These results concur with several reports linking oxidative stress and recurrent miscarriage.[14,15] Consistently, the genetic background of the patients was recently explored,[16] and revealed that *ABCB1*, *COMT*, *GPX4*, and *OGG1* present single-nucleotide polymorphisms (SNPs) that are collectively associated with a 1.97 fold increased risk [confidence interval (CI): 1.31-2.97] of recurrent miscarriage. In the latter study, the genes were genotyped in the mother, which is responsible for only half of the genetic anlagen of the fetus. Nevertheless, it suggests that oxidative stress and free radical metabolism defects are linked to recurrent abortion.

More and more interest is now shown on the relations between oxidative and nitrosative stress in the field of reproduction,[17,18] albeit this issue was apparently not yet sys-

tematically explored in RSA. One of the important chemical intermediates for nitrosative stress is the peroxynitrite ion, ONOO⁻, which results from combining free oxygen species with nitric oxide (NO). NO is considered as a major vasodilator molecule, and plays even a crucial role in maintaining vascular health, especially in the context of pregnancy.[19] NO is produced from l-arginine by NO synthases (NOS). Several studies analyzed the polymorphisms of endothelial nitric oxide synthase (eNOS) in recurrent pregnancy losses. A meta-analysis based upon 18 case–control studies, published in 2011,[20] concluded that the Glu298Asp variant of eNOS was indeed associated significantly, albeit moderately, with RSA [odds ratio (OR): 1.37, CI: 1.11-1.69], this significance being observed only when dominant model was used. This masks a strong variation in the studies. For the present review, we screened 10 studies on eNOS published from 2001 to 2011, but focusing sometimes on polymorphisms other than Glu298Asp. Five studies concluded to an effect,[21-25] while five concluded to no effect.[26-30] It is well known that such discrepancies frequently occur in association studies, and clearly, meta-analysis cannot always substitute well-conducted experimental analyses with a sufficient statistical power.[31] To explain the differences observed in the case of eNOS (but it applies equally well to other polymorphisms), one possibility could be related to the geographic origin of the various studies: Amongst the five positive ones, two are from India, one from Korea, one from China, and only one concerns a Caucasian population (Austria[21]). By contrast, the five negative studies are from Greece, Tunisia, Japan, Germany, and Austria and are, therefore, composed of Caucasian populations except the Japanese study. In the 10 studies that we screened, it is also noticeable that on average, the negative studies were smaller (cases 174 ± 104, controls 120 ± 53) than the positive ones (cases 186 ± 93, controls 169 ± 84), which may contribute to losing detection power.

Maternal side

Hyperimmune reactivity

The idea of the existence of strong links between immunity and reproduction is old, and nurtured by the fact that the fetus expresses paternal antigens that should be the target of graft-rejection mechanisms. Several theories have been proposed to explain the mere biological possibility of the pregnancy.[32,33] The links between major histocompatibility complex (MHC) haplotypes and graft rejection has been clearly established long ago.[34]

Autoimmune diseases

The presence of autoantibodies is often associated with an increased risk of pregnancy loss.[35] This issue

has been recently extensively reviewed.[36] The group of Yehuda Shoenfeld showed a significant increase of several autoantibodies in patients with RSA.[37] Amongst other cues linking autoimmunity and pregnancy failure, it is interesting to quote the work of Mecacci and coworkers that explored the links between the detection of thyroid autoantibodies in the plasma and RSA. They analyzed a group of 69 patients (who were pregnant at least 6 months before the study, consisting of 29 RSA, 22 fetal deaths, and 18 preeclampsias), in comparison with a control group of 69 nonpregnant women, matched for age, parity, and gravidity. There was more than threefold increase in antithyroid autoantibodies in RSA patients compared to controls. Results on mice indicate that the detrimental link discovered in these patients may be disconnected from a direct action on major relevant tissues for pregnancy (such as placenta, decidua, or ovary) where the thyroglobulin gene is not strongly expressed.[38]

Once the links between autoimmunity and pregnancy failure are established, since this review focuses on genetics, the major issue is to collect relevant information on genetic variants leading to an increased risk of autoimmune reactions that could influence pregnancy maintenance. One exemplary study making this link is provided by a work published in 2011 by Salmon and coworkers.[39] In this study, a cohort of 250 patients suffering from systemic lupus erythematosus (SLE) or expressing antiphospholipid antibodies (APL Ab) was analyzed with respect to genes of the complement cascade, since complement modifications have recurrently been shown to deregulate the angiogenic balance in the placenta.[40] Amongst these patients, 17% suffered from preeclampsia (roughly threefold more than expected in the general population). While pregnancy loss was not the principal topic of the study, there are very strong cues to the idea that preeclampsia and pregnancy loss share common genetic bases, especially due to alleles increasing the risk of thrombophilia (see below).[41,42] In the last study, comparing 279 thrombophilic patients to 796 control patients, it was shown in thrombophilic patients (i.e. harboring risk alleles for coagulation factors *F5* rs6025 and *F2* rs1799963) that treatment with low-molecular-weight heparin (LMWH) during a new pregnancy attempt decreased the risk of fetal death recurrence and preeclampsia simultaneously.

In the Salmon study of 2011, which mainly focused on complement factors and preeclampsia, mutations in *MCP* and *CFI* were found to be increased in patients. The complement is classically considered to act along two major pathways (classical and alternative) [Figure 1]. Both pathways induce the hydrolysis of C3 into C3a and C3b. The latter cleaves C5 into C5a and C5b, leading eventually to formation of the membrane attack complex (MAC). Membrane cofactor protein (MCP) and complement factor I (CFI) are cofactors of the system: MCP is a transmembrane protein and CFI is a serine protease able to cleave and inactivate
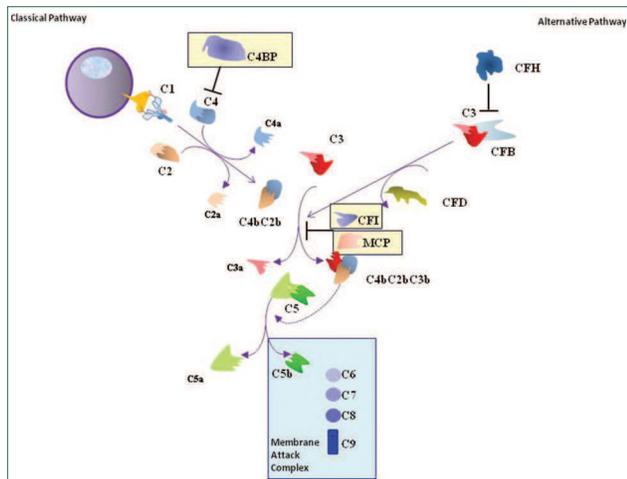
Figure 1: A simplified picture of the two major pathways of the complement cascade (the lectin pathway is not represented). In yellow boxes are presented the genes that have been analyzed for their putative impact on RSA through genotyping populations and that are discussed in this review. Interestingly, the polymorphisms that were studied generally concern genes having an inhibitory action on the complement cascade.

C3b and C4b that are needed for further steps leading to the final constitution of the MAC. MCP A304V corresponds to a variant displaying 50% reduced activity in the control of C3b deposition by the alternative pathway.[43] In addition, and in direct link to RSA, the authors discovered a mutation (K32N) in MCP in an SLE patient with RSA and preeclampsia. This mutation induces a 50% decrease in C4b binding, compared to wild type. This variant also induces a decreased CFI-mediated cleavage activity on C4b. MCP, also known as CD46, had been tested in RSA genetic studies as early as in 1991[44] using the restriction fragment length polymorphism (RFLP) approach. In 1992, a study from Finland, using RFLP as well, discovered associations between C4 variants and tumor necrosis factor (TNF) variants with an increased risk of RSA.[45] Since the two genes are separated by ~400 kb, it is improbable that the authors discovered a mere effect of linkage disequilibrium (LD), but rather a process where the two genes are independently involved through their specific functions in regulating complement/coagulation cascades (case of C4a polymorphisms) and inflammation (in the case of TNF). The same team published in 1992 a study on 58 RSA couples, which did not reveal significant association with TNF variants, strengthening the idea of a necessary allelic association between C4 and TNF variants to observe a strong phenotype.[45] More recent studies carried out in India concur with the idea that MCP/CD46 is a genetic risk factor for abortion,[46] but again with a limited sample (44 patients). A contradictory study was published in 2011 based upon a larger population (75 RSA and 115 controls), which could not put in evidence an effect of the A304V mutation.

More recently, a genetic analysis of three complement pathway inhibitors, MCP/CD46, CD55, and C4 binding protein (C4BP), based on systematic exon sequencing of these factors was performed.[47] Overall, 192 patients and 192 controls were sequenced in this thorough study. A heterozygous, non-synonymous mutation was found in C4BPB and some others in C4BPA (R240H), previously shown to decrease the co-factor activity of C4BP in degrading C3b. All the variants of C4BP were studied *in vitro* to evaluate their capacity of modulating the degradation of C3b and C4b, showing an important reduction of these biochemical capabilities compared to the wild type variant when the R120H variant was used, as well as significant effects of I126T and I224T.

"Wrong" HLA programming

The human MHC system or human leukocyte antigen (HLA) is the "identity card" of humans.[48] It is divided into three groups of genes on chromosome 6 called Class I (HLA-A, -B, -C, -E, -F, -G), Class II (HLA-DP, -DQ, -DR, -DM, -DO), and Class III (including the *TNF* and other genes encoding proteins involved in inflammatory processes). A long time ago, it was shown that the placental structures do not present the same MHC molecules than the rest of the body[49,50] and that increased homozygosity in mothers lead to pregnancy disorders, supposedly by decreasing the chances of allele sharing with the father.[51] As early as in 1992, Takakuwa and coworkers analyzed HLA-DQ antigens by polymerase chain reaction (PCR)-RFLP. This early exploration did not yield significant results,[52] but did not discourage further efforts in investigating this issue. In fact, the Takakuwa team did later find associations between specific DR alleles (DRB1*0403 and DRB1*0410, and later DRB1*1502) and DQ alleles with recurrent miscarriage.[53] Later on, similar increased risks were associated with specific DP alleles, such as DPB1*04.[54,55] By contrast, DP alleles appeared not to have a significant effect in the study of Sagot and coworkers.[56] In 1992, Christiansen and coworkers genotyped DR and DQ by RFLP on 152 patients with RSA and 210 controls from Denmark, and concluded to a significant effect of specific genotypes such as DRw17, DQw2, as well as other specific DQ genotypes.[57-59] Similar involvement of the MHC Class II genes was confirmed in 1993 in the Finnish population.[60] More recent studies in Chinese patients suggest that some DQ alleles are risk factors as well.[61-63] A recent study confirmed the involvement of HLA-DR and -DQ variants to be associated with increased RSA risk in the Brazilian population.[64] Therefore, the association of specific Class II MHC alleles with increased miscarriage seems quite robust. Overall, unfortunately, mechanistic insights from these abundant genetic/epidemiological data are absent.

For Class I genes, this absence of mechanistic ideas on the biological variant function is not totally similar, at least for HLA-G, referred to as an immunosuppressive molecule,[65] and for which several polymorphisms were associated with RSA, as reviewed by Dahl and coworkers.[66] HLA-G belongs to the subclass Ib, encompassing HLA-E, HLA-F, and HLA-G, and is highly expressed on trophoblast cells, which are much less polymorphic than the Class I genes [HLA-A and HLA-B are present in all the tissues except on the trophoblast cells, while HLA-C is expressed at a low level on the trophoblast and has indeed been associated with pregnancy disorders, such as preeclampsia and miscarriage, depending on the allelic complement of the killer cell inhibitory receptor (KIR) harbored by the maternal uterine Natural Killer (uNK) cells].[67,68] A very interesting feature of HLA-G is that upon specific alternative splicing events, the soluble forms HLA-G5, HLA-G6, and HLA-G7 are released and may play a long-range immunomodulatory function. HLA-G is now considered as a reliable marker of the success of *in vitro* fertilization (IVF).[69] In 1997, HLA-G was tested to see if it was playing a putative role in the materno-fetal immunological dialog, but without taking into account any specific polymorphism.[70] In 1999, a consistent study evaluating the impact of an intron 4 polymorphism was published by Yamashita and coworkers.[71] A non-synonymous substitution in exon 4 did not display significant correlations with pregnancy disorders.[72] Nevertheless, the situation with regard to HLA-G started to change with a study using sophisticated mathematical tools and suggesting strongly that further investigation on HLA-G involvement in RSA was needed.[73] In 2002, a Scandinavian study focused on a 14-bp polymorphism present in the 3′ UTR of the gene (generating an Ins/Del in the non-translated 8th exon) and found that, curiously, in RSA couples, the 14-bp deletion was more abundant in men while the 14-bp insertion was more abundant in women.[74] Later, the same team showed that the homozygous presence of the 14-bp sequence of exon 8 in women was significantly associated with infertility, combining the risk of unsuccessful IVF treatments and of RSA, when the two phenotypes were considered together.[75] On the contrary, a study of Indian patients revealed an increase of + 14 bp/ 14 bp heterozygotes in RSA women, and in another study, some significantly different haplotypes in terms of frequency between the two groups of women were observed.[76] In a more recent article, paternal lymphocyte alloimmunization therapy (PLAT) was positively evaluated for treating 42 RSA patients. Thirty-eight became pregnant in the following year and 27 babies were born. Interestingly, a 100% presence of HLA-G*010401 was seen in the 11 abortion cases, while it was present in only 51.9% of the cases that were a success.[77] In 2006, a Chinese team could not find the association between HLA-G and RSA;[78,79] also, a Polish study failed to do so,[80] albeit the team suggested

that nonsignificance was probably due to the small sample size.[81] Two other negative studies were published – one in 2010 that analyzed 143 RSA and 150 control couples in the Indian population[82] and another one in 2012 on an Iraqi population of 50 RSA and 50 control women.[83] By contrast, Xue *et al.* discovered an excess of heterozygotes in Chinese RSA women,[84] while another more recent study from China indicated an increased number of +14 bp homozygotes in the RSA women.[85] Near the 14-bp Ins/Del, two polymorphisms (T1570VC and C1594A) were detected and found to be associated with RSA risk;[86] T1570C was in LD with the cases but not with the controls. Other LD in the Class I region were shown.[87] The comprehensive analysis of HLA-G sequence in the context of RSA was carried out by systematic sequencing of 238 cases and 233 RSA in Caucasian women. Twelve SNPs differed between the two groups of patients, while the authors also confirmed the increased risk brought by the 14-bp insertion.[88] This concurred with the results of two Brazilian studies, the first one analyzing 60 RSA couples and 60 controls[89] and the second one analyzing the outcome of IVF procedures which found an increase of allele HLA-G*01:03:01 in the cases of implantation failures. This story was interestingly refined in 2012, with the idea of a sex-dependent increased risk, when the mother was + 14 bp/+14 bp homozygote and presented with HLA Class II alleles restricting HY immunity. In this case, at the second pregnancy in the case of a boy child, there was a significant decrease in birth weight and an increased risk of miscarriage.[90] Most explanations about this influence of the Ins/Del on pregnancy rely on the idea of differential stability of the mRNA encoding HLA-G and decreasing its concentration. Therefore, HLA-G expression level also appears as an important factor, an idea illustrated by the study of Ober and coworkers in 2003, associating HLA-G polymorphisms and recurrent pregnancy loss unambiguously.[91] In this study, the analysis of the novel variant -725C/G in the HLA-G promoter uncovered an association with miscarriage (OR: 2.76, 95% CI: 1.08-7.09; $p = 0.035$). Interestingly, the G generates a CpG site that is methylated, suggesting a possible epigenetic effect in the mechanisms of recurrent pregnancy loss, and substantiates the idea of potential impact of DNA methylation in reproduction disorders.[92] Recently, and concurrent with this idea, more mechanistic experiments were carried out on trophoblast from first-trimester placentas of voluntarily terminated pregnancies. Immunotolerogenic treatments applied to the cells (dexamethasone, hydrocortisone) were able to modulate HLA-G expression positively, but the basal level of HLA-G was systematically lower in RSA patients.[93]

Since HLA is a relatively small chromosome region (<3 Mbp), LD between Class I and Class II can be expected and was indeed shown, for instance, in the locus encompassing the three Class II DRB1*03-DQA1*05-DQB1*02, and

the Class I G*01010 2. G*010102 is the HLA-G haplotype that encompasses the 14-bp sequence variant.[94] This result could put forward the idea that only one of these genes is actually modulating implantation success, according to the alleles displayed by the patient.

A possible member of the dialog between HLA-G (either soluble or presented by trophoblasts) and uterine natural killer (NK) cells (which are major actors of vasculogenesis in pregnancy) is the KIR family. A genetic study showed, however, that the KIR repertoire was not different between controls and patients, albeit the KIR2DL4 genotypes differed significantly ($p = 0.03$).[95]

Bad adjustments of the cytokinic dialog

Immunity depends strongly on the presence of circulating molecules secreted by the different lymphocyte families. An over-simplistic vision of T cells is the idea of the existence of a tolerogenic versus aggressive balance, materialized as Th1 [inflammatory, secreting, for instance, interleukin (IL)-2 and interferon-gamma (INFγ)] and Th2 (tolerogenic, secreting, for instance, IL-4, IL-5, IL-6, IL-10, and IL-13). Pregnancy was appreciated as a process where Th2 lymphocytes were overpowering Th1 lymphocytes. This prompted geneticists to find associations between cytokine-encoding genes and pregnancy loss.

TH2 pathway

IL-10 polymorphisms were evaluated to this respect as early as in 2001,[96] but failed to pinpoint links between a G > A polymorphism in the promoter (−1082) and a risk of RSA for a population of 38 Finnish patients and 131 ethnically matched controls. A more systematic study of the IL-10 gene promoter analyzed together −592C/A, 819C/T, and −1082A/G in strong LD, in 350 RSA cases and 200 controls,[97] showing that the −592A/ 819T/ 1082A haplotype was more frequent in cases than in controls (OR: 4.01, 95% CI: 1.83-7.95). To note, some negative studies (absence of genetic association found) can result from insufficient polymorphism of the SNP analyzed. For instance, for IL-10, Warren and coworkers reported on the comparison between the −1082A/G variant and a microsatellite present in the promoter of IL-10, and could associate microsatellite alleles but not the SNP to cervical insufficiency, a cause of late miscarriage.

IL-6 variants were studied in RSA versus control patients unsuccessfully in 2003.[98] However, this study was contradicted by Saijo and coworkers who discovered a protector effect of GG genotypes at the −634C-->G promoter polymorphism of IL-6 in the Japanese population.[99] This association was confirmed in a Chinese sample of 162 RSA patients and 156 controls.[100] The same study did not find significant differences in IL-1β-31T, -511T, and + 3954T between RSA patients and controls.

Interleukin 1 receptor antagonist (IL1RA; an immunomodulator encoded by the *ILRN* gene) alleles were also suggested as at risk for RSA,[101] with strong OR values for the effect of a specific allele, *2 (OR: 7.4, CI: 2.9-10.8). Consistent with the idea of an effect of this gene, a study comparing 37 Finnish women with RSA and 800 donors showed an increased frequency of a rare allele (IL1RN*3, OR: 5.6, 95% CI: 1.5-19.0; $p = 0.006$).[102] However, negative studies for IL1RN were also reported in various populations.[103-105] IL1R1 (the receptor of IL-1 with Th1 effects) variants were also devoid of significant effects in a 2011 study.[106]

Always in the Th2 cascade, Il-4 was unsuccessfully explored in 2004 in a Japanese population through genotyping a specific number of repeat variations in a variable number tandem repeat (VNTR) polymorphism.[107]

TH1 pathway

In 2001, Reid and coworkers assessed TNF and IL1B alleles as risk factors for RSA, and did find a trend for association with a TNF variant.[108] Il1RB was also studied for an exon 5 polymorphism (+3953), while the serum levels were followed in parallel in 131 RSA patients and 68 controls, and no significant difference could be found.[109] Later, the same group studied a C/T polymorphism in the IL1B promoter (−511), together with M235T of AGT (SERPINA8) and Glu298Asp of eNOS (NOS3), which was unsuccessful again. However, Wang and coworkers identified IL1B -31T and IL1B -511C to be associated with a history of RSA[110] in two cohorts totaling 262 RPL and 144 controls. This latter study also showed that trophoblasts treated by peripheral blood mononuclear cells (PBMC) of patients harboring the IL1B -511C variant presented an increased INFγ production. INFγ genetic polymorphisms may also be directly involved as suggested by an Argentine study based upon 41 RSA and 54 control women. The authors analyzed variants of INFγ, TNFα, IL-6, IL-10, and transforming growth factor-beta (TGFβ), and found an association between INFγ 874A→T and RSA. Another study analyzed together, though unsuccessfully, the allelic distribution of Il-10 (−1082G > A), TNFα (−308G > A), and INFγ (874T > A) alleles in a group of 43 patients and 73 controls of Caucasian ethnic background.[111] TNFα (−308 G/A) was, nevertheless, found to be significantly associated with RSA in a sample of Saudi women (65 cases vs 65 controls), although the same study failed to put forward associations with Il-6 and Il-10 polymorphisms.[112]

Amongst the proinflammatory cytokines, Il-21 variants were recently evaluated through genotyping a four SNP haplotype (rs9992580/rs4833837/rs2055979/rs13143866) on 235 RM and 235 controls, and strong risk and protective haplotypes could be identified, such as G-A-A-A (OR: 4.02)

and T-G-C-G (OR: 0.09), respectively.[113] Since these haplotypes were present in 11.7% of the patients and 18.1% of the controls, they are not devoid of clinical interest.

Cytokines and immune cells dialog

Links between interleukin signaling and the immune system are numerous. For instance, Il-12 and IL-18 are involved in uterine vascular development through the regulation of uterine NK cells.[114,115] Promoter gene polymorphisms were analyzed for these factors in 125 RSA cases and 136 controls[116] and did not demonstrate any significant association. However, a more recent study of 282 patients and 283 controls from Saudi Arabia on Il-18 promoter haplotypes reconstructed from genotyping four SNPs (rs1946519/rs187238/rs360718/rs360717) showed several haplotypes associated with reduced IL-18 levels and increase RSA risk.[117] A more recent study extended to a Tunisian population the analysis of the same haplotype of four SNPs of Il-18 [−656C/A (rs1946519), −137G/C (rs187238), −119A/C (rs360718), and −105G/A (rs360717)].[118] A-G-A-A and C-G-A-A were associated with an increased risk of RPL (OR: 20.73 and 5.23, respectively), and C-G-A-G conferred a protection (OR: 0.29). Overall, these allelic combinations are found in 54% of the controls and 35% of the patients, which give them an important potential predictive value.

Contribution of meta-analyses

Overall, meta-analyses may help to clarify the issues of the impact of cytokine polymorphism and RSA, such as that of Bombell and McGuire,[119] which evaluated studies on *TNFα*, *IFNγ*, *IL1B*, *Il-6*, and *IL-10* and concluded that *IL-1B* (−31T, at risk, OR: 2.12, CI: 1.04-4.33, from two studies) and *IL-6* (−634G, OR: 0.22, 95% CI: 0.09-0.57, but from one study only) had significant effects. In 2009, another meta-analysis concluded on the effect of the *IL-10* genotype.[120] A report combining experiments and meta-analyses of *IL1B* and *IL1RN* VNTR on a sample of Indian women did not find the association of variants of these genes with RSA.[121] A similar meta-analysis concluded the absence of significant effect of IL-6 (rs1800795) and IFNγ (rs2430561), but comforted the idea of an association of *IL-10* −1082A/G with the risk of RSA (rs1800896).

Thrombophilic disorders

There is an interesting and well-documented literature on the links between pregnancy loss and vascular diseases of pregnancy, particularly preeclampsia. Preeclampsia is a major disease of pregnancy, characterized by a gestational increase of maternal blood pressure and proteinuria.[122,123] The etiology of preeclampsia remains largely unknown; however, a possible scenario is that in its genetic forms, alterations of the coagulation cascades could be a cornerstone of the disease. Collectively, defects leading to increased

coagulation can be assimilated to thrombophilia (also called hypercoagulopathy, an abnormal increase in the propensity of the blood to clot). The coagulation cascade is a complex series of biological events that ought to be tightly controlled [Figure 2]. At the center of this cascade is thrombin (or coagulation factor II, F2), an enzyme able to convert fibrinogen into fibrin, the major constituent of the blood clot. Prothrombin, the precursor of thrombin, is cleaved and thus activated through the action of coagulation factor X (F10). Despite commonalities in coagulation disorders between preeclampsia and RSA, specific genetic anomalies have rarely been found in common between the two disorders,[124] with the possible exception of angiotensin converting enzyme (ACE). In this gene, there is a well-documented insertion/deletion polymorphism (I/D) for which a recent meta-analysis concluded that there was a significant association of the D allele with recurrent miscarriage.[125] In this analysis, 11 studies with 3357 individuals were analyzed. It led to the most significant discrepancy (DD vs II, homozygote model) and the relative risk was estimated at 1.81 (CI: 1.29–2.66; $p = 0.003$). ACE D allele was also associated with preeclampsia (in a recent study[126]).

Overall, the subject of a genetic basis for recurrent pregnancy loss has been strongly associated with thrombophilic disorders, with major scientific contributions.[127] This early study was probably the first to systematically analyze polymorphisms located in FV Leiden (F5), *MTHFR*, *F2* (prothrombin), on a limited number of patients (67) and controls (232). In this seminal paper from the group of Irene Cetin, *FVL* and prothrombin mutations were found to be associated with a triple risk of pregnancy loss. Overall, the existence of a thrombophilic state seems a recurrent issue in RSA, as reviewed several times.[128-130] Following these early discoveries, thrombophilia appeared as a general risk
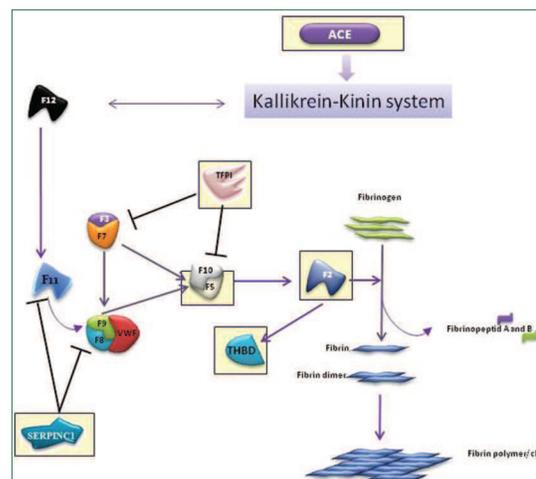

Figure 2: A simplified picture of the coagulation cascade. Genes that were mainly studied for their putative relation with RSA are represented in yellow boxes.

factor for pregnancy; logically, there have been several studies throughout the world, aiming at identifying polymorphisms in coagulation cascade factors encoding genes, in the context of recurrent miscarriage. Recently, Cao and coworkers carried out an extensive analysis of factors known to be involved in thrombophilia.[131] They genotyped six polymorphisms in *SERPINC1* (G786A), *THBD* (C1418T), *TFPI* (T-33C), *FVL* (G1628A), *F2* (prothrombin, A19911G), and *ANXA5* (G76A) using mass array spectrometry. This screen allowed identifying a significant effect only of thrombomodulin (THBD) from 94 cases and 169 controls. Interestingly, a large part of the genes studied were common to those of another study from Brazil.[132] The latter article focused upon 117 RSA and 264 control patients, who were analyzed for protein C (*PROC*), at two SNPs (A2418G and C2405T), *SERPINC1* (G786A, common to the previous study), *THBD* (C1418T, common to the previous study), and *TFPI* (T-287C, C-399T, and T-33C, common to the previous study), *FVL* (G1691A), and *F2* (G20210A); therefore, five genes and three SNPs were common to both studies. Univariate logistic regression revealed an effect of *SERPINC1* and *TFPI* variants only. The *P* value for *THBD* was estimated at 0.661, and did not even reveal a trend. It is interesting to note, however, that the T-33C variant, common to both studies was not significant in any of them. In the study of Cao, *SERPINC1* did not display any trend as well. Therefore, there are clear discrepancies between the two studies for *THBD* and *SERPINC1*. Analyzing these two situations may, nevertheless, be illustrative of specific pitfalls in this type of association studies, the major one certainly being the ethnic origin of the patients: Chinese in one case, Brazilian and multiethnic in the other. This leads to strong discrepancies in the basic allelic frequencies of each of the common SNPs in the two studies [Table 1]. In

Table 1: Rare allele frequencies in two populations studied for RPL in the context of coagulation cascades

	Cao (2013) Chinese	Guerra-Shinohara (2012) Brazilian	P value
SERPINC1 (G786A)			
Cases	0,298	0,137	5,14 10^{-5}
Controls	0,311	0,087	3,97 10^{-17}
THBD (C1418T)			
Cases	0,293	0,132	4,96 10^{-5}
Controls	0,207	0,116	2,4 10^{-4}
TFPI (T-33C)			
Cases	0,101	0,35	2,41 10^{-9}
Controls	0,107	0,324	2,55 10^{-13}

The polymorphisms that were significantly different between controls and RSA patients are in bold. The *P* values refer to significant statistical differences in allelic frequencies between the two populations assessed by a χ^2 contingency test, independent of the potential effect in terms of pregnancy loss, RPL: Recurrent pregnancy loss, *SERPINC1*: ???, *THBD*: ???, *TFPI*: ???

this table, the alleles that were significantly different in one of the populations are marked in gray. This type of analysis demonstrates highly significant discrepancies in the allelic frequencies between the two populations, as well as in cases and in controls.

Whole genome approaches

In terms of genetic determinism, the novel existing tools of genome wide association studies have recently allowed to identify genomic regions putatively encompassing genes involved in pregnancy success or pregnancy loss. However, so far, only two papers have been published dealing with such approaches.[133,134] The premises of the latter study were provided by the observation of an increased risk of miscarriage in sisters sharing identical HLA variants.[58,135] This is consistent with the increased risk of recurrent pregnancy loss exhibited by patients homozygous for the 14-bp insertion in the 8th exon of HLA-G, as previously described. The study of 2011 confirmed the increased risk of recurrent miscarriage in siblings by analyzing 244 patients and 381 full siblings, of whom 23 had more than two miscarriages and three had two siblings with more than two miscarriages. The average risk of miscarriage in the control population was almost stable (10-15%) between 15 and 32 years of maternal age, rising steeply after this age. But at any age, the risk was between 1.5- and 2-fold higher in the siblings than in the reference population (25.3 vs 13.1%). By a genetic study using only 30 small nuclear families and ~60,000 SNPs, suggestive linkages on subpopulations could be detected for SNPs in the genes *FHIT* (3p14.2), *FAM154A* (9p22.1), *PDE2A* (11q13.4), and *GRIK2* (6q16.3) [Logarithm of the Odds (LOD) scores were between 2.5 and 3]. The other genome wide study published in 2010 by Li and coworkers identified three regions (6q27, 9q33.1, and Xp22.1) that were not the same.[133] In fact, again, the dissimilar origin of the population under scrutiny (Han Chinese) can be one of the causes of this phenomenon. Also, it was a pilot study performed on a very limited number of samples, with medium marker resolution (430 microsatellites). To the best of our knowledge, a high-density SNP analysis (with 250,000 or 500,000 SNPs) has not been performed yet, while such an analysis could improve the detection.

Conclusions

As shown before, the genetic study of pregnancy loss focused overall on a limited number of genes, which have been analyzed in numerous human populations. Following these observations, two hypotheses can be proposed: either (i) The major genes have already been found and duly studied and they explain much of the genetic bases of RSA or, on the contrary, (ii) this phenotype is so complex that many genes

with small effects can cause the disease, and the systematic study of some of them results from a very partial understanding of the causes underlying RSA. One such example is methylene tetrahydrofolate reductase (MTHFR). This enzyme, which is ubiquitously expressed, is responsible for the synthesis of 5-methyltetrahydrofolate. This molecule is the main methyl donor allowing conversion of homocysteine to methionine. In 1997, a Dutch team decided to study the 677 C→T mutation, shown as a risk factor for coronary artery disease in particular.[136] This study, published in *The Lancet*,[137] compared 197 patients who suffered from two or more consecutive miscarriages, with 113 matched controls and with a larger population of 1250 women. The author concluded that the 677 TT genotype was associated with a ~2-3 fold increased risk of recurrent miscarriage. Following this study, 39 other articles dealing with MTHFR and RPL can be found in Pubmed, up to the year 2013, amongst which one is a recent meta-analysis,[131] combining 677 C→T with another polymorphism (A1298C). This last work focused on 3559 cases and 5097 controls and concluded that the T allele had a mod erate negative effect (CT + TT genotypes, OR: 1.68, 95% CI: 1.32–2.13; $p < 0.0001$), the effect being stronger in East Asian populations.

It is interesting to note that almost all the studies attempted to identify significant variants by genetically analyzing a limited range of individuals (almost always less than 300). This means that rare variants cannot be found despite a clear effect. An exception to this is the identification of the JAK2 V617F mutation when a huge sample of 3496 pairs of women was analyzed.[138] With such a powerful structure, it was pos-

sible to detect a very significant increased risk of fetal loss but with low allelic frequencies of the V617F mutation (1.06% in cases vs 0.20% in controls, OR: 4.63, CI: 1.76-12.2). The risk was even higher in women with embryonic losses than in those with fetal loss. This observed effect of the low frequency of the risk variant could logically be very difficult to reproduce in smaller samples, while the biological interest of the study remains intact in terms of understanding the pathophysiology of RSA. Another possible approach to discover new genes is to use information gleaned from model species, following genome scans. This was recently performed in an interspecific recombinant congenic collection of mice followed during gestation by ultrasonography,[139] which recently allowed to propose human alkaline phosphatase polymorphisms to be associated with increased or decreased risk of pregnancy loss.[140] In addition, various studies on mice demonstrated the importance of several pathways in this model species as well; this is the case for the complement system and its modulator genes. For instance, mouse models have provided a solid base of the importance of complement activation in successful pregnancies (C3 being a pivotal molecule), as well as its regulation by Crry, sometimes in relation with autoimmune diseases.[40,141-145] Therefore, while the topic of the present review was human disease, it is clear that much can be understood mechanistically using animal models, especially rodents.

Different genetic cascade defects can result in pregnancy loss [Figure 3]. Very few studies on humans revealed functional insights on the events ending in pregnancy termination. Some pieces of this puzzle may definitely be missing today, but it would be of great importance to move to more mechanistic models to better understand the genetic bases of abortion.
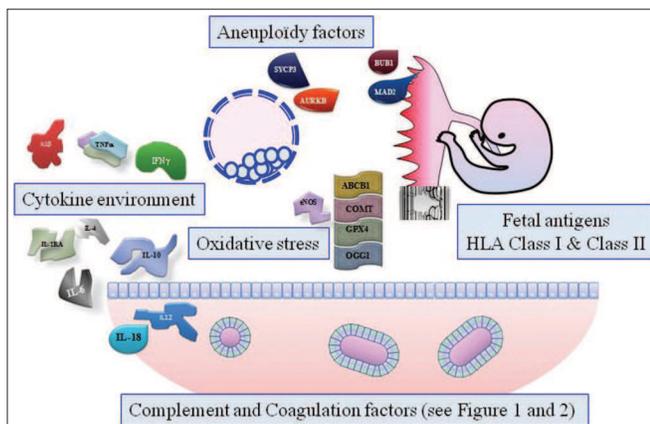

Figure 3: A simplified and not exhaustive graphic summary of the factors that may genetically influence pregnancy success. Several factors are more thoroughly described in the text. Even though the subject of the review is, strictly speaking, about pregnancy loss and, therefore, rather later stages than the blastocyst (presented in blue), early defects at this stage may allow implantation, but will trigger failure later on. Clearly, the propensity of the blastocyst to release free radicals, for instance, can foster defective immunomodulation, excessive inflammation leading to pregnancy loss, or to preeclampsia in less-severe cases.

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