Polymorphisms of the angiotensinogen gene, the endothelial nitric oxide synthase gene, and the interleukin-1β gene promoter in women with idiopathic recurrent miscarriage

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Interleukin (IL)-1β, angiotensinogen (Agt), and endothelium-derived nitric oxide synthase (eNOS) are thought to be involved in idiopathic recurrent miscarriage (IRM). We investigated the correlation between IRM and common polymorphisms in Agt, Nos3 and IL-1β genes: one polymorphism in the promoter region of the IL-1β gene, one in exon 2 of the Agt gene, and one in exon 7 of the Nos3 gene. A total of 130 women with a history of IRM and 67 healthy control women were included in the study. Genotyping for the C/T transition at position −511 in the promoter region of IL1B, for the single base M235T polymorphism of Agt, and for the missense Glu298Asp variant of Nos3 was performed using PCR, an allele-specific oligonucleotide hybridization assay, and pyrosequencing, respectively. Allele and genotype frequencies of all polymorphisms were similar among women with IRM and controls. Between women with primary and secondary recurrent miscarriages, no statistically significant differences between allele and genotype frequencies were observed. Despite promising experimental data, our data fall short of showing any significant association between a variant of the promoter region of IL1B, the M235T polymorphism of Agt, and the Glu298Asp missense variant of Nos3 and the occurrence of IRM.

Key words: angiotensinogen/idiopathic recurrent miscarriage/interleukin-1β/Nos3/polymorphism

Introduction

Recurrent miscarriage, defined as three or more consecutive pregnancy losses before 20 weeks gestation, affects 0.5–2% of pregnant women (Anonymous, 2001). A series of aetiological factors, including uterine anomalies, maternal/paternal balanced translocations, luteal phase defect, hyperprolactinemia, and hyperhomocysteinaemia have been identified for this condition (Anonymous, 2001). In up to 50% of cases, however, the exact underlying pathophysiological mechanisms remain undetermined (Anonymous, 2001). An inherited component for these idiopathic recurrent miscarriages (IRM) has been suggested.

Another aetiological concept of IRM refers to the role of the maternal inflammatory response versus the semi-allogenic fetus (Hill et al., 1995; Shaarawy and Nagui, 1997). A reduction of pro-inflammatory cytokines by a T-helper cell (TH)-1/TH-2 balance shift, seen in healthy pregnant women, is distinctly absent in women experiencing IRM (Wegmann et al., 1993; Hill et al., 1995).

Interleukin (IL)-1β is an essential pro-inflammatory cytokine, produced by monocytes, macrophages, and epithelial cells. Secretion of IL-1β leads to a pro-inflammatory cascade, including production of tumour necrosis factor (TNF)-α, interferon (IFN)-γ, IL-2, and IL-12. The gene encoding IL-1β is located within a 40 kb region on human chromosome 2q14.2 (Bioque et al., 1995). In-vitro data using mononuclear cells from healthy individuals have demonstrated that a polymorphism at position −511 in the promoter region of IL1B is associated with an increased capacity of IL-1β production (Santtila et al., 1998). Given the pro-inflammatory nature of IL-1β, it is reasonable to consider IL1B a candidate gene for IRM.

Another biological pathway thought to lead to IRM involves genes regulating vascular tone. Besides the well described role
of angiotensinogen (Agt) in the renin–angiotensin system (RAS) regulating vasopressor, electrolyte, and fluid homeostasis (Stroth and Unger, 1999), increasing evidence has suggested a role for Agt in embryonic development. Agt, angiotensin (AT) II, and ATII receptors (AT₁ and AT₂) were found to be expressed in human embryos during pregnancy (Schuetz et al., 1996) and are involved in organ development (Gelly et al., 1991; Schuetz et al., 1996; Price et al., 1997). In a mouse model, we have previously shown that mice deficient for the angiotensinogen gene have an increased risk of early embryonic waste (Temperet et al., 2000).

The common M235T polymorphism of the Agt gene located on chromosome 1q42-43 (Caulfield et al., 1994) has been investigated in a wide variety of diseases including hypertension (Jeunemaitre et al., 1997) and pre-eclampsia (Ward et al., 1993). A significant association between the M235T polymorphism and elevated plasma Agt levels has been ascertained (Rotimi et al., 1997). This has been interpreted as evidence for a biochemical mechanism linking DNA variation in the Agt gene with the described phenotype.

A direct involvement of nitric oxide (NO) in the pathogenesis of IRM has also been proposed. NO is released during the conversion of L-arginine to L-citrulline by three isoforms of nitric oxide synthase (NOS), i.e. endothelial (e)NOS, inducible (i)NOS, and neuronal (n)NOS (Moncada and Higgs, 1993). NO is known to mediate vascular smooth muscle relaxation and has been implicated in the development of endothelial damage, hypertension, coronary spasm, and myocardial infarction (Nakayama et al., 1999; Lind et al., 2000). Experimental data in mice and previously published results in humans point to a crucial role of NO in the course of pregnancy (Hefer et al., 2001). With respect to IRM, experimental data in mice have shown that lipopolysaccharide (LPS)-induced abortion is mediated by placental NO production (Athanassakis et al., 1999). Pharmacological inhibition of NO release by aminoguanidine successfully rescues LPS-induced abortion (Haddad et al., 1995).

The exon 7 Glu298Asp missense variant of Nos3 on chromosome 17p11-q11 (Xu et al., 1994) is a common polymorphism and is associated with various diseases such as myocardial infarction (Shimasaki et al., 1998), placental abruption (Yoshimura et al., 2001), and pre-eclampsia (Yoshimura et al., 2000). Altered intracellular cleavage of the Glu298Asp missense isoform of eNOS has been suggested to be responsible for pathophysiological processes leading to impaired endothelial function (Tesoro et al., 2000).

Based on their biological functions, the IL1B, Agt and Nos3 genes can be seen as candidate genes for IRM. In this study, we attempted to establish an association between the polymorphism at position −511 in the promoter region of IL1B, the M235T polymorphism of the Agt gene, and the missense Glu298Asp variant of the Nos3 gene and the occurrence of IRM in a Caucasian population. We hypothesized that the incidence of these polymorphisms would be increased among women with IRM.

Materials and methods

Patients

The diagnosis of IRM was based on a documented history of at least three spontaneous, consecutive miscarriages before 20 weeks gestation with the same partner. To avoid confounding by ethnicity, only white Caucasian women were included in the study and control groups. To avoid confounding by genetic admixture, only women whose parents were of the same ethnicity were included in the study and control groups. A total of 130 women were included in the study group. These women underwent a standard diagnostic work-up to rule out a verifiable cause of the recurrent miscarriages. Diagnostic procedures included hysteroscopy, paternal and maternal karyotype, cervical cultures for chlamydia, ureaplasma, and mycoplasma, a comprehensive hormonal status, and evaluation of antiphospholipid syndrome with IgM and IgG anticardiolipin antibody assessment and lupus anticoagulant testing. Among these women, primary recurrent miscarriage was defined as no history of a pregnancy carried beyond 20 weeks gestation. Secondary recurrent miscarriage was defined as a history of at least one pregnancy carried beyond 20 weeks gestation. The control group consisted of 67 women with at least two live births and no history of miscarriage. All control women were postmenopausal to rule out possible future miscarriages after inclusion in the study. Written informed consent was obtained from participating women.

Genotyping

DNA was obtained from blood using the Puregene System (Gentra Systems, Research Triangle Park, NC, USA) or the Qiagen System (QIAmp DNA Blood Midi Kit; Qiagen, Hilden, Germany). The extracted DNA was stored at 4 °C until analysed.

IL1B

A transition from cytosine to thymine at position −511 in the promoter region of the IL1B gene was searched for using the following PCR strategy: oligonucleotide primers (forward: 5′-TGGCATGTCTCTGTTCACTC-3′ and reverse: 5′-GGTTAGGAATCTCCTCCACTT-3′) were used to amplify a 304 bp genomic DNA fragment. PCR conditions comprised an initial denaturing step at 95 °C for 1 min, followed by 30 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s, and a final extension at 72 °C for 5 min. A restriction fragment length polymorphism (RFLP) was resolved on a 3% agarose gel after digestion with Ava I (New England Biolabs Inc., Beverly, MA, USA). Subjects who carry the wild type allele (allele C) generate a PCR product with a recognition site for the restriction enzyme, whereas those with the mutant allele (allele T) generate a 190 bp and a 114 bp band. The wild type C allele is represented by a 190 bp and a 114 bp band. The mutant T allele is represented by a 304 bp band. Lanes 1, 2 and 3 show homozygous wild type, homozygous mutant and heterozygous patterns, respectively.
generate a PCR product with no recognition site. Using this PCR strategy, allele C generated a 190 bp band and a 114 bp band, while allele T generated a 304 bp band.

**Agt**

Genotyping for the M235T polymorphism of the *Agt* gene was performed using an allele-specific oligonucleotide hybridization (ASO) technique. PCR with genomic DNA as template was used to amplify a 386 bp fragment in exon 2. The forward primer sequence was 5’-AAGGACACGACTGCACCCTCCCGCT-3’. The reverse primer sequence was 5’-GCCAGACCGAGGAGGTGTTGCT-3’. PCR products were then spotted onto two nylon membranes (NEN Life Science Products, Boston, MA, USA) and allowed to dry. The membranes were then placed in 0.4 mol/l NaOH for 15 min to denature and neutralized in a 2× standard saline citate (SSC), Tris-HCl solution. The membrane for the 235M allele was then hybridized in a solution containing Church buffer (Parchem Company, Parkersburg, West Virginia, USA), herring sperm DNA, a 35P-end-labelled oligonucleotide probe corresponding to the 235M allele, and a non-radioactive oligonucleotide corresponding to the 235T allele. For the 235T allele, the hybridization method was repeated with labeled 235T probe and unlabelled 235M probe. Oligonucleotide probe sequences were: 235M, 5’-TCCCTGATGGGAGCCAG-3’; 235T, 5’-TCCCTGACGGGAGCCAG-3’. Membranes were sealed in a bag containing the corresponding hybridization solution and then placed in a water bath at 67°C. The temperature was then lowered over 6 h to 34°C. Following hybridization, membranes were washed three times at room temperature in 5×SSC for 5 min each and then for 30 min at 34°C in 2×SSC. Autoradiographs were used to score (present/absent) each allele separately. We further validated our ASO technique by sequencing four samples with an ABI 377 automated sequencer. Results were in agreement; two samples were homozygous 235T, one sample was heterozygous, and one sample was homozygous 235M.

**Nos3**

Pyrosequencing was used to genotype women for the eNOS Glu298-Asp missense variant which results from a G to T mutation in exon 7. Twenty-five nanograms of DNA was used as template for all PCR amplifications. The reaction mixture included 5 pmol of both sense and antisense primers, 250 µmol/l dNTP (ViennaLab, Vienna, Austria), 4.0 µl of 10× Amplification Buffer (10 µmol/l Tris–HCl, pH 9.0, 50 mmol/l KCl, 0.01% (w/v) gelatin, 1.5 mmol/l MgCl₂, 0.1% Triton X-100; ViennaLab) and 1.0 IU Super Taq Polymerase (HT Biotechnology Ltd, Cambridge, UK). PCR was performed on a Perkin–Elmer GeneAmp PCR system 9600. The following genotyping strategy was used: oligonucleotide primers (forward: 5’-CAGGAAACGGTCGCTCCAGACG-3’; reverse 5’-CCATCCACCCAGGTTCAATC-3’) were used to amplify a 148 bp genomic DNA fragment. PCR conditions comprised an initial denaturing step at 94°C for 1 min, followed by 40 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s, and a final extension at 72°C for 5 min. Polymorphisms were detected using a Pyrosequencer PSQ 96 and PSQ 96 SNP Reagent Kit (Pyrosequencer, Uppsala, Sweden). A total of 35 µl of PCR product was used for pyrosequencing according to the manufacturer’s instructions. Five pmol of the sequencing primer 5’-CCAGGGCCCGCAATC-3’ were applied to detect the corresponding polymorphisms.

**Statistical analysis**

Differences in the frequencies of the *IL1B*, *Agt* and *Nos3* alleles in the study and control groups were analysed by χ²-test. The odds ratio (OR) was used as a measure of the strength of the association between allele frequencies and IRM. All P-values were two-tailed and 95% confidence intervals (CI) were calculated. P < 0.05 was considered statistically significant. A sample size calculation showed that with an expected frequency of the mutant allele of *IL1B*, *Agt* and *Nos3* of 40, 30 and 30%, respectively, 130, 120 and 120 samples are needed to detect a difference of 25% between groups at a significance level of 0.05 with a power of 85%. We used the SPSS statistical software system (SPSS 10.0; SPSS Inc. Chicago, IL, USA) to do the calculations.

**Results**

Characteristics of women with IRM are shown in Table I. Distributions among women with IRM and controls for all polymorphisms were in Hardy–Weinberg equilibrium.

**IL1B**

Allele frequencies among women with IRM and controls were 56.9 and 58.2%, respectively, for allele C (wild type), and 43.1 and 41.8%, respectively, for allele T (mutant) (Table II). No association between allele T and the occurrence of IRM was found (P = 0.89, OR 0.95). Genotype frequencies were not significantly different between the study group (C/C: 22.3%; C/T: 69.2%; T/T: 8.5%) and the control group (G/G: 20.9%; G/T: 56.7%; T/T: 13.4%) (P = 0.9). Between women with primary and secondary recurrent miscarriages, no statistically significant difference between genotypes was observed (P = 0.3).

**Agt**

Allele frequencies among women with IRM and controls were 52.8 and 53.8%, respectively, for allele M (wild type) and 47.2 and 46.2%, respectively, for allele T (mutant) (Table III). No association between allele T and the occurrence of IRM was found (P = 0.95, OR 0.96). Genotype frequencies were not significantly different between the study group (M/M: 27.2%; M/T: 51.3%; T/T: 21.5%) and the control group (M/M: 30.3%; M/T: 47%; T/T: 22.7%) (P = 0.85). Between women with primary and secondary recurrent miscarriages, no statistically significant difference in the distribution of genotypes was observed (P = 0.3).

**Nos3**

Allele frequencies among women with IRM and controls were 68.1 and 67.9%, respectively, for allele G (wild type), and 31.9 and 32.1%, respectively, for allele T (mutant) (Table IV). No association between allele T and the occurrence of IRM was found (P = 0.92, OR 1.01). Genotype frequencies were not significantly different between the study group (G/G: 46.2%; G/T: 43.7%; T/T: 10.1%) and the control group (G/G: 48.4%; G/T: 39.1%; T/T: 12.5%) (P = 0.9). Between women

<table>
<thead>
<tr>
<th>Gene variants in recurrent miscarriage</th>
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<tbody>
<tr>
<td><strong>Table I. Characteristics of women with idiopathic recurrent miscarriage</strong></td>
</tr>
<tr>
<td>Parameters</td>
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<tr>
<td>Median age (years) at miscarriage evaluation (range)</td>
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<tr>
<td>Median no. of miscarriages (range)</td>
</tr>
<tr>
<td>Median no. of live-births (range)</td>
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<tr>
<td>No. of primary aborters (%)</td>
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<td>No. of secondary aborters (%)</td>
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</tbody>
</table>
Table II. Allele frequencies and genotypes of the *IL1B* promoter polymorphism among women with idiopathic recurrent miscarriage and controls

<table>
<thead>
<tr>
<th>Genotypes</th>
<th>Women with IRM (n = 130, 260 alleles)</th>
<th>Controls (n = 67, 134 alleles)</th>
<th>P^a</th>
<th>χ^2</th>
<th>Odds ratio (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genotypes</td>
<td>C/C 29 (22.3) 20 (29.9) 0.3 0.9(^b) 1.7(^b) (0.7–4.3)</td>
<td>C/T 90 (69.2) 38 (56.7)</td>
<td>T/T 11 (8.5) 9 (13.4)</td>
<td>C 148 (56.9) 78 (58.2) 0.9 0.02(^b) 0.9 (0.6–1.5)</td>
<td>T 112 (43.1) 56 (41.8)</td>
</tr>
</tbody>
</table>

Values in parentheses are percentages.
^aχ^2 test.
^bCalculation was performed for C/C and C/T versus T/T.
IRM = idiopathic recurrent miscarriage; CI = confidence interval.

Table III. Allele frequencies and genotypes of the *Agt* polymorphism among women with idiopathic recurrent miscarriage and controls

<table>
<thead>
<tr>
<th>Genotypes</th>
<th>Women with IRM (n = 130, 260 alleles)</th>
<th>Controls (n = 67, 134 alleles)</th>
<th>P^a</th>
<th>χ^2</th>
<th>Odds ratio (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genotypes</td>
<td>M/M 35 (26.9) 20 (29.9) 0.9 &lt;0.001(^b) 1.1(^b) (0.5–2.1)</td>
<td>M/T 67 (51.5) 32 (47.8)</td>
<td>T/T 28 (21.5) 15 (22.4)</td>
<td>M 137 (52.7) 72 (53.7) 0.9 0.008(^b) 0.9 (0.6–1.5)</td>
<td>T 123 (47.3) 62 (46.3)</td>
</tr>
</tbody>
</table>

Values in parentheses are percentages.
^aχ^2 test.
^bCalculation was performed for M/M and M/T versus T/T.
IRM = idiopathic recurrent miscarriage; CI = confidence interval.

Table IV. Allele frequencies and genotypes of the *Nos3* polymorphism among women with idiopathic recurrent miscarriage and controls

<table>
<thead>
<tr>
<th>Genotypes</th>
<th>Women with IRM (n = 130, 260 alleles)</th>
<th>Controls (n = 67, 134 alleles)</th>
<th>P^a</th>
<th>χ^2</th>
<th>Odds ratio (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genotypes</td>
<td>G/G 60 (46.2) 32 (47.8) 0.9 0.03(^b) 1.2(^b) (0.5–3.1)</td>
<td>G/T 57 (43.8) 27 (40.3)</td>
<td>T/T 13 (10.0) 8 (11.9)</td>
<td>G 177 (68.1) 91 (67.9) 0.9 &lt;0.001(^b) 1 (0.6–1.6)</td>
<td>T 83 (31.9) 43 (32.1)</td>
</tr>
</tbody>
</table>

Values in parentheses are percentages.
^aχ^2 test.
^bCalculation was performed for G/G and G/T versus T/T.
IRM = idiopathic recurrent miscarriage; CI = confidence interval.

With primary and secondary recurrent miscarriages, no statistically significant difference between genotypes was observed (P = 0.5).

Discussion

This is the first report on common polymorphisms of the promoter region of *IL1B*, and of the *Agt* and *Nos3* genes in women with IRM. Based on their biological functions these genes can be seen as candidate genes for the disease. Our data, however, fail short of showing any association between the examined polymorphisms and the occurrence of IRM.

As for diabetes (Froguel and Velho, 2001), hypertension (Lee et al., 2000), and pre-eclampsia (Roberts and Cooper, 2001), multiple genes encoding for proteins involved in various biological pathways have been reported to be associated with IRM. Data are currently available to support a role for thrombophilic genes (Wramsby et al., 2000) and for genes encoding cytokines (Unfried et al., 2001) and biotransformation enzymes (Zusterzeel et al., 2000) in the pathophysiology of
IRM. However, a critical combination of variants necessary or sufficient to cause IRM has not yet been described. The available data provide evidence that the development of IRM is complex and is regulated by multiple genetic pathways.

During physiological pregnancy, the maternal immune response is selectively suppressed to avoid rejection of the semi-allogenic embryo/fetus (Wegmann et al., 1993). While TH-2 type immunity is believed to contribute to successful pregnancy, TH-1 type-dependent immunity to trophoblast has been shown to be associated with IRM (Wegmann et al., 1993; Hill et al., 1995; Jenkins et al., 2000). Murine studies (Wegmann et al., 1993) and elevated elevated cytokine levels, including IL-1β, in humans (Shaarawy and Nagui, 1997) indicate that dominance of TH-1 type-dependent cytokines, such as IL-1, is incompatible with successful pregnancy. Our study, however, falls short of determining a significant effect of the IL1B genotype on IRM. We found no association between IL1B genotype or allele frequencies and IRM in a representative Caucasian population. These results are in accordance with recent data reported by Reid and colleagues. They examined another IL1B polymorphism in a British population and found no significant difference in the carriage of mutant alleles between women with IRM and controls (Reid et al., 2001).

Thrombophilic mutations in the factor II, factor V, and methylene tetrahydrofolate reductase (MTHFR) genes have been shown to be involved in intrauterine growth restriction (Glueck et al., 2001), pre-eclampsia (Glueck et al., 2001), IRM (Wramsby et al., 2000), and late fetal loss (Martinelli et al., 2000). Agt can be regarded as both a vascular and a thrombophilic gene, and has been shown to be involved in hypertension, and thrombophilic processes in mouse models and humans (Kim et al., 1995; Winkelmans et al., 1999).

The transformation of the spiral arteries to large vessels of low resistance has been reported as a crucial event for the viability of the embryo in early pregnancy (Lyall et al., 1999). Therefore we assumed that, based on its known biological functions, Agt may have a certain role in the pathophysiology of IRM as shown in mice (Tempfer et al., 2000). Our results, however, fall short of verifying our hypothesis. Nevertheless, we cannot rule out the possibility that polymorphisms of Agt other than M235T could be associated with the disease.

Given the aetiological role of NO deficiency in hypertension, vasospasm, haemorrhage, and infarction, it is reasonable to speculate that carriers of a Nos3 polymorphism and subsequently reduced NO serum levels (Tsukada et al., 1998) could be at increased risk for impaired placental perfusion and infarction. An impaired oxygen and nutrient supply might compromise the ability of the embryo/fetus to resist maternal alloimmune rejection in early pregnancy. However, our data indicate that the investigated exon 7 Glu298Asp missense variant of Nos3 does not confer an increased risk of developing IRM.

Ethnic variation and genetic admixture need to be considered in an evaluation of the genetic background of IRM (Hartl and Clark, 1992). Thus, we made efforts to reduce error in the interpretation of our results by only considering white Caucasian women, whose parents were of the same ethnicity in the study and control groups. Another concern relates to the selection of a proper control group. Some other studies investigating women with recurrent miscarriage used age-matched controls to compare genotype frequencies. This strategy does not rule out future abortions among control women. To avoid this possible bias, all control patients in our series were post-menopausal at the time of blood sampling. Of note, definitions of IRM have varied between studies. Some previous studies have defined IRM as three or more miscarriages before 20 weeks of gestation (Meinardi et al., 1999; Carp et al., 2001), whereas others have not (Kutteh et al., 1999). We opted for defining recurrent miscarriage as three or more miscarriages before 20 weeks of gestation in order to provide comparable data. It has to be kept in mind, however, that miscarriages in early pregnancy might be aetiologically different than those occurring early in the second trimester.

It is a limitation of our study that we cannot specify at what level an increase or decrease in protein expression regulated by the investigated genes could affect the risk of developing IRM. With respect to Nos3, impaired NO production might impair early pregnancy at the gonadal level, placental level, or at the level of the systemic vasculature. Whether the pathophysiological mechanisms result in impaired transformation of the spiral arteries, impaired placental function and infarction, or whether other effects might be involved needs to be further elucidated. Furthermore, it can be argued that evaluating polymorphisms only in the mother does not reflect pathophysiological processes leading to IRM. It has to be stated that the aim of our present study was to evaluate whether the presence of maternal polymorphisms increases the risk of developing this condition. Based on the biological functions of the three genes investigated and the negative results obtained, we doubt that investigating these polymorphisms in the embryonic/trophoblast tissue would provide any additional information.

Although experimental and preliminary data generated in our laboratory have pointed to a critical role of IL-1β, Agt and eNOS in IRM, our study falls short of showing any significant association between the polymorphism at position −511 in the promoter region of IL1B, the M235T polymorphism of the Agt gene, and the Glu298Asp missense variant of Nos3 and the occurrence of IRM. Our results indicate that these polymorphisms should not be included in future studies involving panels of various polymorphisms. However, we cannot exclude the possibility that other polymorphisms of IL1B, Agt and Nos3 are associated with the disease and might be clinically useful as markers to assess a woman’s risk for IRM.

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