Article

Association between genetic polymorphisms in cytokine genes and recurrent miscarriage – a meta-analysis

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Abstract

A meta-analysis of association studies was performed to assess whether the reported genetic polymorphisms in cytokine genes are risk factors for recurrent miscarriage (RM). The electronic PubMed database was searched for case–control studies on immunity-related genes in RM. Investigations of a single polymorphism/gene involvement in RM reported more than five times were selected. Aggregating data from seven case-control studies on –308/tumour necrosis factor-α polymorphism, the odds ratio (OR) for RM was 1.1 (0.87–1.39) if the polymorphism was considered under a dominant genetic model. In six studies on –1082/interleukin-10 (IL-10) polymorphism, the OR under a dominant model was 0.76 (0.58–0.99), and under a recessive model the OR was 0.90 (0.71–1.15). In five case–control studies on –174/IL-6 polymorphism, the OR for RM under a recessive model was 1.29 (0.69–2.40). The results show a statistically significant association with RM for the –1082/IL-10 genotype.

Keywords: *cytokines, gene, meta-analysis, polymorphism, recurrent miscarriage*

Introduction

Recurrent miscarriage (RM) could be defined as the loss of two or more, or three or more clinically detectable pregnancies with no reference to the week of gestation. When defined as two or more pregnancy losses it occurs in approximately 5% of all couples, while defined as three or more spontaneous abortions, it affects approximately 1% of the population (Stirrat, 1990; Coulam, 1991; Roy Choudhury *et al.*, 2001). The causes can be divided into inherited and acquired, embryological and maternal, but more than 50% of cases of RM remain idiopathic. Maternally driven causes include coagulation, autoimmune, endocrine disorders and endometrial defects. Dysregulated immunity has been suggested as a possible cause of idiopathic RM (Chaouat *et al.*, 2002; Laird *et al.*, 2003, 2006; Wilson *et al.*, 2004; Beydoun *et al.*, 2005). The involvement of the immune system in pregnancy and in RM could be analysed at different levels, e.g. the level of the mother's global immune system, the specificity of her immune system regarding paternal antigens of the fetus or the specialized maternal immune system in the placenta, the latter two being difficult to investigate. Therefore, the investigation of the role of immune system in the aetiology of RM is based on the analysis of immune cells and immune mediators/cytokines in the peripheral blood of the mother, as well as of genes coding for them. These investigations provide a deeper insight into the maternal causes of RM.

T-helper (Th) cells play a central role in the cytokine network. Although oversimplified, maternal immune system and cytokine production responsible for successful pregnancy are predominantly anti-inflammatory, belonging to the Th2 subpopulation (Wegmann *et al.*, 1993; Makhseed *et al.*, 1999), which produce the cytokines interleukin 3 (IL-3), interleukin 4 (IL-4), interleukin 5 (IL-5), interleukin 6 (IL-6), interleukin

10 (IL-10) and interleukin 13 (IL-13), whereas the Th1 proinflammatory response, comprising cytokines interleukin 2 (IL-2), interferon (IFN)-γ, tumour necrosis factor (TNF)-α and interleukin 3 (IL-3), is associated with RM (Jenkins *et al.*, 2000, Lim *et al.*, 2000; Makhseed *et al.*, 2001). The concentrations of cytokines are influenced by polymorphisms in cytokine genes (Laird *et al.*, 2003). Various studies have been performed on the association of cytokine gene polymorphisms and RM with inconclusive results. Meta-analysis of genetic association studies on cytokines provides a powerful tool in epidemiology and evidence-based medicine to overcome limitations of individual association studies such as inappropriate study design, sample size, spurious positive association and lack of statistical power, and inter-study heterogeneity.

A meta-analysis of case–control association studies was performed on RM and genetic polymorphisms in cytokine genes TNF-α, IL-10 and IL-6, as these genes are most frequently investigated in individual association studies.

Materials and methods

Search strategy

The electronic PubMed MEDLINE (http://www.ncbi.nim.nih.gov/pubmed/ accessed 18 February 2009) database was searched up until September 2007 for studies on candidate genes in recurrent miscarriage. The investigation was initially based on the medical subject headings terms: recurrent spontaneous abortion (RSA), recurrent pregnancy loss (RPL), recurrent miscarriage (RM) and recurrent fetal loss (RFL), in combination with genetic polymorphism and mutation. All genetic association studies evaluating involvement of any genetic polymorphisms in RM were registered. Besides association studies, review articles, systematic reviews, and meta-analysis articles were also considered. An additional search of the articles was performed through the references cited in identified articles, through the link 'related articles' offered in the PubMed database, and through the references of review articles. The special target was the identification of case–control studies on immunity-related genetic polymorphisms and RM. Therefore, an additional investigation was performed on the basis of the medical subject heading terms RSA, RPL, RM, and RFL, in combination with 'cytokine' and 'cytokine gene polymorphisms'. After identification of the studies reporting on these, an additional article search was performed in PubMed with new medical subject heading terms: RSA/RPL/RM/RFL and the name of the individual candidate gene detected on primary investigation. The article search was performed independently by three authors (IM, SO, NP).

Study selection

After the search through the keywords 'RSA/RPL/RM/RFL' and 'genetic polymorphism', 264 papers were identified (**Figure 1**). Among them were the papers on the involvement of genes

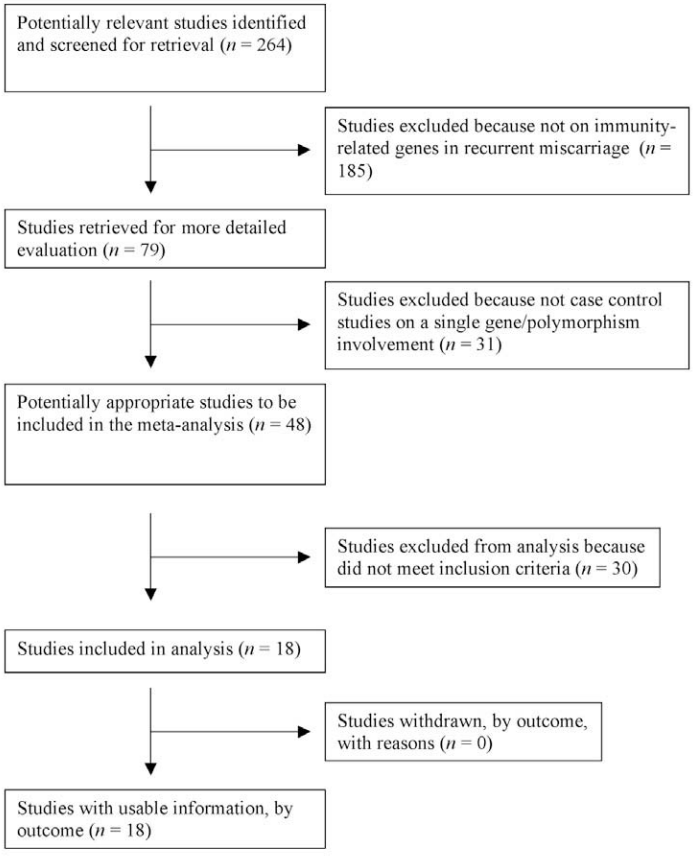

Figure 1. QUOROM statement flow diagram.

coding coagulation factors, vasoactive substances, metabolic factors, cytokines and regulatory genes in RM, systematic reviews of these genetic polymorphisms, as well as protein and chromosomal studies in RM. Only the studies analysing the association between genetic polymorphisms in immunity-related genes and RM were further considered. Studies on human leukocyte antigen sharing in RM were not evaluated because they were reviewed in 2005 (Beydoun *et al.*, 2005). Further selection of these studies was based on the following inclusion criteria: retrospective case–control studies with diagnostic criteria of RM and genotype frequencies reported. Finally, only polymorphisms investigated in at least five studies were included in the meta-analysis. On the basis of these criteria, it was necessary to exclude studies on genetic polymorphism in interleukin-1β, interleukin-1 receptor antagonist, IL-4, interleukin-12, interleukin-18, transforming growth factor (TGF)-β1, cytotoxic T-lymphocyte-associated protein (CTLA) 4, TNF-β, and IFN-β, in RM, as these genetic polymorphisms were reported fewer than five times. Only the studies dealing with single polymorphisms in the *TNF-β gene*, the *IL-10* gene (three polymorphisms: –592, –819, and –1082), and the *IL-6* gene (–174, –634), were further considered.

Data extraction

The final review and meta-analysis were performed on seven studies on –308 polymorphism in the *TNF*-β gene (Babbage *et al.*, 2001; Baxter *et al.*, 2001; Reid *et al.*, 2001; Daher *et al.*, 2003; Pietrowski *et al.*, 2004; Prigoshin *et al.,* 2004; Kamali-Sarvestani *et al.*, 2005), six studies on –1082 polymorphism in the *IL-10* gene (Babbage *et al.*, 2001; Karhukorpi *et al.*, 2001; Daher *et al.*, 2003; Prigoshin *et al.*, 2004; Kamali-Sarvestani *et al.*, 2005; Zammiti *et al.,* 2006) and five case-control studies on –174 polymorphism in the IL-6 gene (Saijo *et al.*, 2001; Daher *et al.*, 2003; Unfried *et al.*, 2003; Prigoshin *et al.*, 2004; Von Linsingen *et al.*, 2005).

For each single polymorphism/RM analysis, the following data were extracted: author, country and year of publication, ethnicity and continent of patients and controls, RM definition, patients' and controls' mean age if reported, and clinical exclusion criteria. Only case-control studies with an explicit definition of exposure were included, i.e. genetic polymorphism and the data on genotype frequencies, and defined outcome, women with three RM; however, a few studies on women with two or three RM were also included. For each polymorphism/RM analysis, even if already performed, the odds ratio (OR) was calculated again. The odds of an event are calculated as the number of events divided by the number of non-events; the odds ratio is calculated by dividing the odds in the exposed group by the odds in the control group, the OR having superior mathematical properties than relative risk, and being more appropriate for meta-analyses.

In the analysis, if the available data allowed, the OR were calculated under both assumptions: the supposed effect of a polymorphism if dominant (the effect already present when the polymorphism mutant type present in heterozygous state: patients with the combination wild type/mutant type plus patients with combination mutant type/mutant type are compared with patients carrying wild type/wild type genotype), and if recessive (the effect present only if the mutant type polymorphism is homozygous: patients with genotype combination mutant type/mutant type are compared with patients with the combination mutant type/wild type plus patients with the genotype wild type/wild type). There is evidence that the investigated gene polymorphisms act as functional polymorphisms, changing the gene product (protein) level, but it has not been elucidated whether the mutant type polymorphisms confer disease susceptibility in different ways when present in homozygous or in heterozygous state (Turner *et al.*, 1997; Fishman *et al.*, 1998; Brull *et al.*, 2001; Reviron *et al.*, 2001; Kilpinen *et al.*, 2001).

Statistical analysis

The selected data were analysed statistically by R programming language (http://www.r-project.org accessed 18/02/09). For each genetic variant study, individual and pooled OR and associated 95% CI were calculated, using fixed-effects model (Mantel–Haenszel method) and random-effects model (DerSimonian–Laird method) (Agresti, 2002; Whitehead, 2002). A P-value <0.05 was considered to be significant. Tests for heterogeneity were performed for each meta-analysis (Q score): a P-value <0.05 was considered to indicate that homogeneity was unlikely (Higgins *et al.*, 2003). For the assessment of publication bias the funnel plot and the Egger regression asymmetry test were used (Egger *et al.*, 1997).

Results

Genetic associations between RM and polymorphism genotypes of the *TNF*-α, *IL-10* and *IL-6* genes were examined. More than five association studies were found for the TNF-β/–308 G→ polymorphism (seven studies), for the IL-10/–1082 G→ polymorphism (six studies), and for the IL-6/–174 G→ polymorphism (five studies).

In all these 18 studies (12 papers), the diagnostic criteria and genotype frequencies were well defined. Since the genotypes were not always separately reported in individual studies, the statistical meta-analysis was performed as follows: for the IL-10 polymorphisms under both the dominant and the recessive genetic model, for the TNF-β polymorphism under the dominant genetic model, and for the IL-6 polymorphism under the recessive genetic model. The genotype distribution among control subjects in each study did not deviate from the expected Hardy–Weinberg equilibrium.

–308/TNF-α polymorphism in RM

The characteristics of the seven analyses on the risk of RM in subjects with the –308 polymorphism in the *TNF*-α gene are summarized in **Table 1**. These studies involved 524 patients and 771 controls.

When comparing homozygous carriers of the mutation plus heterozygous carriers (AA + AG) versus homozygous carriers of the wild type allele (GG), the estimate of the pooled OR based on the random-effect assumption was 1.10 (0.87–1.39). No heterogeneity in genotypic distribution was found (Cochran's Q statistic: chi-squared = 4.07, df = 6; Higgins statistic: I^2 = 0%). No publication bias was detected, using the conservative Egger's regression test of funnel plot asymmetry (t = 1.82, df = 5). **Figure 2** shows the results of individual and summary OR estimates.

–1082/IL-10 polymorphism in RM

The characteristics of the six analyses on the risk of RM in subjects with the –1082 polymorphism in the *IL-10* gene are summarized in **Table 2**. These studies involved 635 patients and 691 controls.

When comparing homozygous mutant type plus heterozygous carriers (AA + GA) versus homozygous carriers of the wild type allele (GG) the estimate of the pooled OR based on the random-effect assumption was 0.76 (0.58–0.99) ($P = 0.04$). No heterogeneity in genotypic distribution was found (Cochran's Q statistic: $\chi^2 = 3.55$, $df = 5$; Higgins statistic: $I^2 = 0\%$). No publication bias was detected, using the conservative Egger's regression test of funnel plot asymmetry ($t = 0.09$, $df = 4$).

When comparing homozygous mutant type carriers (AA) versus heterozygous carriers plus homozygous wild type allele carriers (GA + GG) the estimate of the pooled OR based on the random-effect assumption was 0.90 (0.71–1.15). No heterogeneity in genotypic distribution was found (Cochran's Q statistic: $\chi^2 = 3.55$, $df = 5$; Higgins statistic: $I^2 = 0\%$). No publication bias was detected, using the conservative Egger's regression test of funnel plot asymmetry ($t = 1.11$, $df = 4$). The results of individual and summary OR estimates are shown in **Figure 3a**,**b**.

–174/IL-6 polymorphism in RM

The characteristics of the five analyses on the risk of RM in subjects with the –174 polymorphism in the *IL-6* gene are summarized in **Table 3**. These studies involved 376 patients and 453 controls.

When comparing homozygous mutant type carriers (CC) versus heterozygous carriers plus homozygous carriers of the wild type allele (GC + GG), the estimate of the pooled OR based on the random-effect assumption was 1.29 (0.69–2.40). No heterogeneity in genotypic distribution was found (Cochran's Q statistic: $\chi^2 = 6.86$, $df = 4$; Higgins statistic: $I^2 = 42\%$). No publication bias was detected using the conservative Egger's regression test of funnel plot asymmetry ($t = 0.92$, $df = 3$). The results of individual and summary OR estimates are shown in **Figure 4**.

Discussion

In this systematic review of studies on the involvement of cytokine genetic polymorphisms in RM aetiology, meta-analyses were performed on three genes: *TNF-α*, *IL-10* and *IL-6*, and their respective single polymorphisms, as these had been investigated in more than five studies. The results of the meta-analyses did not demonstrate a significant association between RM and the –308 polymorphism in the *TNF-α* gene when the effect of the polymorphism was considered under a dominant genetic model. In addition, no association was demonstrated for the IL-6/–174 gene polymorphism when it was considered under a recessive genetic model. The results of the meta-analysis of the IL-10/–1082 gene polymorphism involvement in RM showed a significant association, the mutant type allele being associated with RM, when the polymorphism was considered under a dominant genetic model.

Cytokines induce changes in gene expression within their target cells and act as growth and differentiation factors (Parham, 2000). They have many effects on reproduction

Table 1. Characteristics of studies on the association between tumour necrosis factor-α/–308 G→ polymorphism and recurrent miscarriage (RM).

Study, country, Ethnicity	*Cases: number and genotypes*	*Controls: number and genotypes*	*Comments/statistical methods used in the study*[a]
Kamali-Sarvestani *et al.* (2005): Iran, Iranian, Asia	131; AA,AG 14; GG 117	143; AA,AG 21; GG 122	Patient ascertainment/diagnostic criteria appropriate – 3 RM/*P*
Prigoshin *et al.* (2004): Argentina, Argentine Caucasian, America	41; AA,AG 6; GG 35	54; AA, AG 5; GG 49	Patient ascertainment/diagnostic criteria appropriate – 3 RM/*P*
Pietrowski *et al.* (2004): Germany, Central European Caucasian, Europe	168; AA,AG 35; GG 133	212; AA,AG 45; GG 167	–308+ 1 polymorphism; patient ascertainment/diagnostic criteria appropriate – 3 RM/*P*, odds ratio
Daher *et al.* (2003): Brazil, Brazilian Caucasian, America	48; AA,AG 12; GG 36	108 (82f +26 m); AA,AG 19; GG 89	Patient ascertainment/diagnostic criteria appropriate – 3 RM/*P*. Men were among controls
Baxter *et al.* (2001): UK, Caucasian, Europe	76; AA,AG 25; GG 51	138; AA,AG 44; GG 94	Patient ascertainment/diagnostic criteria appropriate – 3 RM. The investigation was performed on couples, not just women
Reid *et al.* (2001): UK, Caucasian, Europe	17; AA,AG 8; GG 9	43; AA,AG 14; GG 29	Patient ascertainment/diagnostic criteria appropriate – 2 RM
Babbage *et al.* (2001): UK, Caucasian, Europe	43 AA,AG; 13 GG 30	73; AA,AG 17; GG 56	Patient ascertainment/diagnostic criteria appropriate – 3 RM/*P*, odds ratio

OR = odds ratio.
[a]If *P* appears in this column, this indicates that statistical analysis was used by the cited authors.

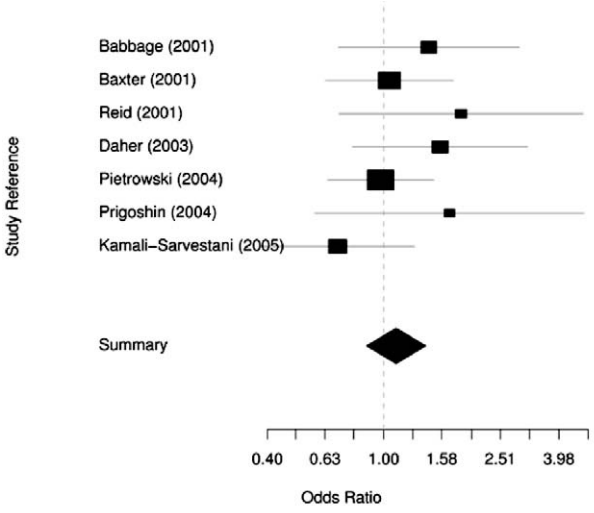

Figure 2. Results of individual and summary odds ratio estimates with 95% confidence interval (CI), random-effect model: –308 G/A/ tumour necrosis factor-α polymorphism – dominant genetic model. The size of the square is proportional to the percentage weight of each study; horizontal lines represent 95% CI.

Table 2. Characteristics of studies on the association between interleukin-10/–1082 G→ polymorphism and recurrent miscarriage (RM).

Study, country, ethnicity	*Cases: number and genotypes*	*Controls: number and genotypes*	*Comments/statistical methods used in the study*[a]
Zammiti *et al.* (2006): Bahrain, Tunisian, North Africa	344; GG 72; GA 185; AA 87	200; GG 39; GA 107; AA 54	Analysed –1082+ 2 other polymorphisms. Patients ascertainment/diagnostic criteria appropriate – 3 RM/*P*, odds ratio
Kamali-Sarvestani *et al.* (2005): Iran, Iranian, Asia	127; GG 24; GA 41; AA 62	130; GG 21; GA 47; AA 62	Analysed-1082+ 2 other polymorphisms. Patient ascertainment/diagnostic criteria appropriate– 3 RM/*P*
Prigoshin *et al.* (2004): Argentina, Argentine Caucasian, America	40; GG 6; GA 21; AA 13	53; GG 9; GA 33; AA 11	Analysed –1082+ 2 other polymorphisms. Patient ascertainment/diagnostic criteria appropriate – 3 RM/*P*
Daher *et al.* (2003): Brazil, Brazilian Caucasian, America	43; GG 11; GA 19; AA 13	104; GG 16; GA 43; AA 45	Patient ascertainment/diagnostic criteria appropriate – 3 RM. Men among controls/*P*
Karhukorpi *et al.* (2001): Finland, Finnish, Europe	38; GG 9; GA16; AA13	131; GG 23; GA 64; AA 44	Patient ascertainment/diagnostic criteria appropriate – 3 RM/*P*
Babbage *et al.* (2001): UK, Caucasian, Europe	43; GG 12; GA 23; AA 8	73; GG 12; GA 41; AA 20	Patient ascertainment/diagnostic criteria appropriate – 3 RM/*P*, odds ratio

OR = odds ratio.
[a]If *P* appears in this column, this indicates that statistical analysis was used by the cited authors

as they are involved in gamete development, implantation, trophoblast invasion, decidualization, placental development and pregnancy immunotolerance (Norman *et al.*, 1996; Clark, 1999; Hales, 2000). Th1 cells are involved in cell-mediated response and delayed-type hypersensitivity; Th1 cytokines produce cytotoxic and inflammatory reactions, and embryotoxic reactions (Hill, 1991). Th2 cells are involved in humoral immunity; Th2 cytokines may prevent maternal Th1 response against the conceptus (Raghupathy, 1997). The maintenance of pregnancy may depend on the type and concentration of cytokines secreted, as they may be protective or harmful to the conceptus. Successful pregnancy has been proposed to be associated with the shift of maternal immune response from proinflammatory Th1 to anti-inflammatory Th2, with fetal loss being associated with the effects of Th-1 type cytokines; these are the conclusions of cytokine investigations in pregnancies in humans and mice.

In this meta-analysis, three cytokines were evaluated: TNF-α produced by Th1 cells, and IL-10 and IL-6 produced by Th2 cells.

TNF-β is a potent proinflammatory cytokine, its circulating concentration being higher in patients with RM (Mueller-Eckhardt *et al.*, 1994; Jenkins *et al.*, 2000; Raghupathy *et al.*, 2000). In the study by Kruse *et al.* (2003), it was suggested that high TNF-β concentrations could, early in pregnancy, be an efficient predictor of RM. Additionally, administration of Th1

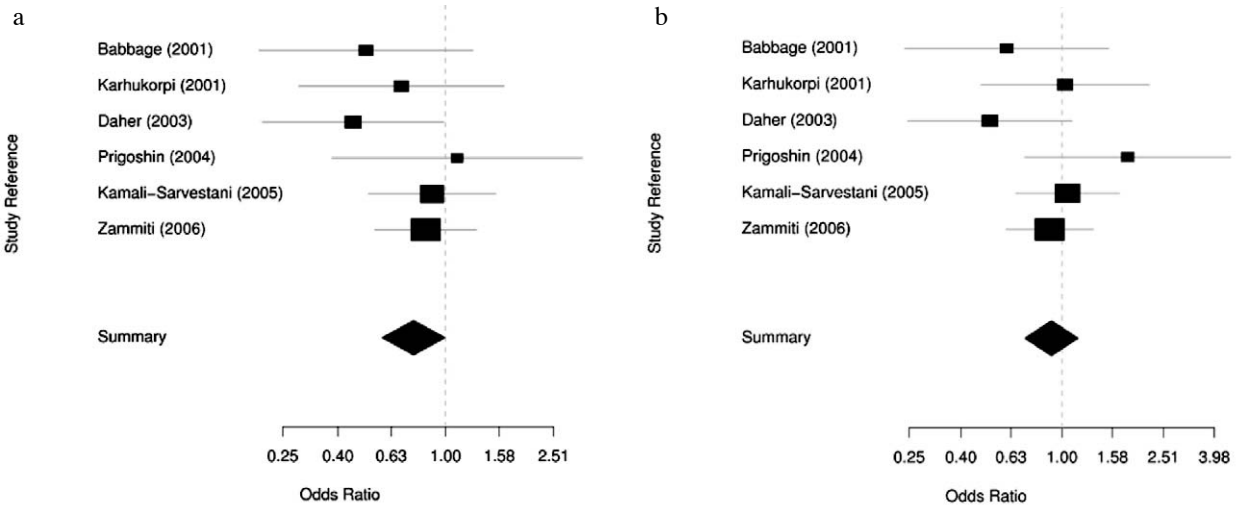

Figure 3. Results of individual and summary odds ratio estimates with 95% confidence interval (CI), random-effect model. (**a**) –1082 G/A/interleukin-10 (IL-10) polymorphism – dominant genetic model. (**b**) –1082 A/G/ IL-10 polymorphism – recessive genetic model. In both cases, the size of the square is proportional to the percentage weight of each study; horizontal lines represent 95% CI.

Table 3. Characteristics of studies on the association between interleukin-6/–174 G→polymorphism and recurrent miscarriage (RM).

Study, country, ethnicity	*Cases: number and genotypes*	*Controls: number and genotypes*	*Comments/statistical methods used in the study*[a]
Von Linsingen *et al*. (2005): Brazil, Brazilian, America	57 (24 + 33)[b]; GG 21; GC 26; CC 10	74; GG 40; GC 31; CC 3	Patient ascertainment/diagnostic criteria appropriate – 2 or 3 RM/*P*
Prigoshin *et al*. (2004): Argentina, Argentine Caucasian, America	38; GG + GC 35; CC 3	54; GG + GC 49; CC 5	Patient ascertainment/diagnostic criteria appropriate – 3 RM/*P*
Unfried *et al*. (2003): Austria, Caucasian, Europe	161; GG 66; GC 72; CC 23	124; GG 43; GC 58; CC 23	Patient ascertainment/diagnostic criteria appropriate – 3 RM/*P*, OR
Daher *et al*. (2003): Brazil, Brazilian Caucasian, America	44; GG + GC 39; CC 5	108 (82f +26 m); GG + GC 99; CC 9	Patient ascertainment/diagnostic criteria appropriate – 3 RM. Men among controls/*P*
Saijo *et al*. (2004): Japan, Japanese, Asia	76 (29 + 47)[b]; GG 76; GC 0; CC 0	93; GG 93; GC 0; CC 0	Analysed –174 + 1 other polymorphism. Patient ascertainment/diagnostic criteria appropriate – 2 or 3 RM/*P*, OR

OR = odds ratio.
[a]If *P* appears in this column, this indicates that statistical analysis was used by the cited authors.
[b]First value in parentheses represents number of patients with 2 RM; second value represents number of patients with three and more RM.

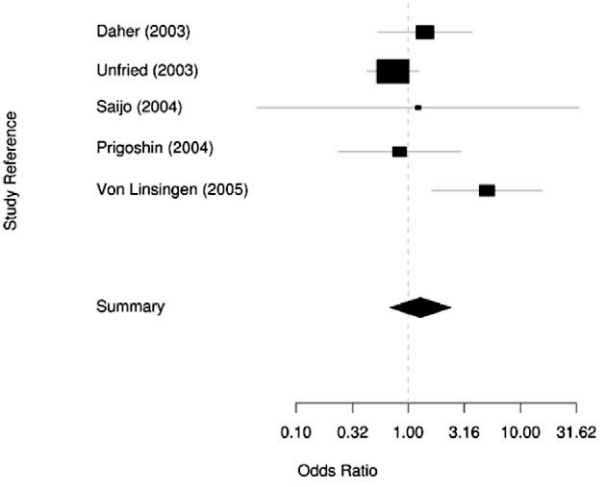

Figure 4. Results of individual and summary odds ratio estimates with 95% confidence interval (CI), random-effect model: –174 G/C/IL-6 polymorphism – recessive genetic model. The size of the square is proportional to the percent weight of each study; horizontal lines represent 95% CI.

cytokines to normal pregnant mice increases fetal resorption (Clark and Croitoru, 2001; Zhu *et al.*, 2005). The biallelic G/A polymorphism at position –308 of the TNF-β promoter has an influence on the TNF-β production: the allele adenine (A) at position –308 is associated with an increased protein serum concentration, therefore enhancing its proinflammatory activity and susceptibility to fetal loss. It is expected, therefore that association studies will reveal an association between the A genotype and RM.

IL-10 plays a key role in Th2 immunity, since it establishes immunosuppressive and anti-inflammatory status during pregnancy; it ensures a Th2 cytokine environment and down-regulates Th1 cytokines (Clark and Croitoru, 2001; Hanlon *et al.*, 2002). Higher concentrations of IL-10 were found in healthy parous women in comparison with women with idiopathic RM (Raghupathy *et al.*, 1999; Jenkins *et al.*, 2000; Daher *et al.*, 2003). Decreased production of IL-10 by decidual T cells in women with RM has been reported (Piccinni *et al.*, 1998). It has been shown that IL-10 production is influenced by the biallelic A/G polymorphism at position –1082 in the promoter region of the gene, adenine (A) allele being associated with a higher, and guanine (G) with a lower production of IL-10 (Turner *et al.*, 1997). Additionally, Hoffmann *et al.* (2001) reported that the GCC haplotype (–1082, –819, –592 respectively) is associated with decreased IL-10 production. Therefore, it is expected that the A genotype is to be found through association studies as a protective factor ensuring successful pregnancy. However, in a recently published study, Mälarstig *et al.*(2008) found that the –1082/IL-10 polymorphism was not a genetic marker of IL-10 in-vivo production, and that the genetic effect of these variants on IL-10 plasma concentrations was relatively small.

Decreased plasma concentrations of IL-6 were found in women with RM in comparison with those with normal pregnancies (Koumantaki *et al.*, 2001). Additionally, decreased expression of IL-6 mRNA was demonstrated in the mid-secretory phase of the menstrual cycle associated with habitual abortion (Von Wolff *et al.*, 2000). The IL-6/–174 G/C biallelic polymorphism alters IL-6 transcription: the IL-6 plasma concentrations are increased in wild type guanine (G) carriers in some studies (Fishman *et al.*, 1998; Reviron *et al.*, 2001), and decreased in others (Brull *et al.*, 2001; Kilpinen *et al.*, 2001).

For the meta-analysis of –308/TNF-β polymorphism involvement in RM, seven studies on seven populations were available. The summary OR demonstrated that combined homozygous plus heterozygous carriers of the mutant allele (dominant genetic model) have a negligible and statistically not significantly increased risk of RM. The interpretation of the recessive genetic model is not possible: each study reported the number of mutant homozygotes and heterozygotes together, both in patients and in controls. In individual case-control studies performed in populations of Caucasian–European origin (three studies in British populations, and one study in Germans, one in Brazilians, and one in Argentineans), and in Iranians, the association between the polymorphism and RM was not demonstrated. Also, in the article by Daher *et al.* (2003) that described a meta-analysis of four individual studies, no statistically significant association was demonstrated. Additionally, when only the populations of European origin were evaluated, no statistically significant association was detected (data not shown).

For the meta-analysis of –1082/IL-10 polymorphism involvement in RM, six studies on six populations were available. The summary OR, considering the polymorphism under a recessive genetic model, did not demonstrate an increased risk of RM in carrier women. However, if considered under a dominant genetic model, an association of the polymorphism with RM was detected, the GG genotype being associated with RM. A similar result was obtained when only the studies on populations of European origin were considered (data not shown). In individual studies performed in Tunisians, Iranians and other Caucasians (four studies), an association between polymorphism and RM was not demonstrated. Besides an individual analysis of Brazilian Caucasian women with RM, Daher *et al.* (2003) performed a meta-analysis of three individual studies comprising 124 patients and 308 controls: an association was demonstrated between the polymorphism and RM, the GG genotype increasing its risk. There are controversies in the reports on IL-10 concentrations in women with successful pregnancy versus women with RM (Vives *et al.*, 1999; Jenkins *et al.*, 2000; Bates *et al.*, 2001); discrepancies have been reported in cytokine production in the circulation and on the maternal–fetal interface (Vives *et al.*, 1999). However, the IL-10 impact on maintaining pregnancy remains questionable, for two additional reasons: firstly, the controversial results could reflect the differences in allele distribution and effects of polymorphisms among different ethnic groups (Laguila Visentainer *et al.* 2008); secondly, in two individual studies, by Kamali-Sarverstani *et al.* (2005) and by Zammiti *et al.* (2006), an association was found between RM and –592 C/A polymorphism in the *IL-10* gene (the polymorphism being in strong linkage disequilibrium with –1082 polymorphism), and with IL-10 haplotype. Considering these findings together with the results obtained in the study by Costeas *et al.* (2004), who found that the balance of IL-10 expression with other Th2/Th3 cytokines is crucial for successful pregnancy, there is definitely a need for further investigation of the role of IL-10 in RM.

For the meta-analysis of –174/IL-6 polymorphism involvement in RM, five studies on five populations were available. Since in two studies genotypes were not reported separately, only the role of the polymorphism under a recessive genetic model was investigated and the summary OR demonstrated that women carriers of the homozygous mutant allele did not have a significantly increased risk of RM. In individual case-control studies performed in Caucasians (one in Argentineans, one in Brazilians and one in Austrians), the Japanese, and in Brazilians of non-specified origin, the association between the polymorphism and RM was not demonstrated. In the Japanese population not a single mutant allele was detected either in patients or in controls. No statistically significant association was detected when populations of European origin only were evaluated apart as a sub-meta-analysis (data not shown).

In the present meta-analyses, attempts were made to avoid general limitations originating from original papers, such as selection bias or publication bias. Study selection was rigorous: only studies with clear diagnostic RM criteria and with reliable standardized molecular genetics methods reported were considered. Most of the studies defined RM as three or more pregnancy losses, but in three reports the patients with two RM were also included. In all these studies, the diagnostic criteria were otherwise appropriate, and so were the exclusion criteria, thus, the participants, interventions and outcome measures

among studies were similar and comparable. In addition, a possible selection bias due to non-considering of non-English-language studies was avoided, as no such study was retrieved during the database search. The search was comprehensive and systematic, performed by three authors, thus publication bias was also avoided. In all meta-analyses, funnel plots were symmetrical and the Egger tests were not significant, indicating a low probability of publication bias. The OR under a fixed-effect model and under a random-effect model were calculated. No significant interstudy heterogeneity was observed. All results of individual association studies were re-examined, and individual OR calculations (dominant or recessive genetic model) were performed in each study regardless of the statistical method previously applied. In all meta-analyses, the aggregated number of cases was distinctively greater than the number of patients in any single study, allowing a more precise estimate of risk. Such an approach makes it possible to increase statistical power and narrow identification of causative genes.

Although in the meta-analysis evidence was found only of the IL-10/−1082 gene/polymorphism involvement in RM, but not of the TNF-α/−308 and the IL-6/−174 genotypes, immunological and immunogenetic factors remain crucial for pregnancy maintenance. They should be further studied in particular models, at particular times in pregnancy to enable insight not only into the level of the mother's global immune system, but also into specificities of her immune system regarding paternal antigens of the fetus, and into specificities of her placental immune system. Considering the complexity of cytokine cell origins and their immunological effects, their interactive function not only between themselves, but also with HLA molecules and immune cells, and knowing that they act locally, it is likely that there is not a single but a complex cytokine influence on a possible mechanism of RM.

Regarding methodological approach to genetic association studies, further systematic studies on a single informative and functional polymorphism are recommended, preferably on greater cohorts of well-defined patients and exact RM definition, haplotype analysis of multiple polymorphisms within a gene, as well as studies of combinations of polymorphisms in several cytokine genes. Besides cytokine gene polymorphisms, other genes and environmental influences should also be investigated.

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