Symposium: Embryo implantation failure and recurrent miscarriage

Future directions of failed implantation and recurrent miscarriage research

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Abstract

Recurrent implantation failure is today the major reason for women completing several IVF/intracytoplasmic sperm injection attempts without having achieved a child, and is probably also the explanation for many cases of unexplained infertility. Most causes of recurrent miscarriage are still poorly elucidated, but from a theoretical point of view recurrent implantation failure and recurrent miscarriage are suggested to have partly overlapping causes. Recent research has indeed documented that both syndromes can be caused by the same embryonic chromosomal abnormalities and the same maternal endocrine, thrombophilic and immunological disturbances. Consequently, many treatments attempting to normalize these abnormalities have been tested or are currently used in women with both recurrent implantation failure and recurrent miscarriage. However, no treatment for the two syndromes is at the moment sufficiently documented to justify its routine use. In this review, an overview is given regarding present knowledge about causes that may be common for recurrent implantation failure and recurrent miscarriage, and suggestions are put forward for future research that may significantly improve understanding and treatment options for the syndromes.

Keywords: *cytokines, implantation failure, NK cells, preimplantation genetic screening, recurrent miscarriage, spontaneous abortion*

Introduction

During 25 years of IVF treatment and research, the major progress has been in improving stimulation protocols and fertilization procedures, and the vast majority of IVF/intracytoplasmic sperm injection (ICSI) cycles now result in the transfer of high quality embryos. However, in the same period only marginal improvement of the implantation and pregnancy rates per transfer has been achieved. With regard to recurrent miscarriage (RM), it is also uncertain whether any real improvement of the overall success rate has been achieved, at least when comparing the period 1968–1977 with 1987–1991 (Plouffe *et al*., 1992). There is thus an urgent need for more and better research on the topics of recurrent implantation failure (RIF) and RM.

The question can be posed: why consider research in failed implantation and RM in a common paper? Are the two conditions not distinct entities with separate aetiologies and pathophysiologies? Indeed, no definite answer can be given. Although exact percentages are impossible to assess, it has been estimated that approximately 30% of embryos are lost at the preimplantation stage, 30% are lost after the embryos implant in the uterus but prior to the missed period, and thus only detectable (in some cases) by a positive serum human chorionic gonadotrophin (HCG), and 10% are lost after the missed period and referred to as clinical miscarriages (Macklon *et al*., 2002). From a theoretical point of view, it must be expected that there are aetiologies that are common for preimplantation losses, non-detectable post-implantation (occult) losses, biochemical pregnancies and clinical losses and aetiologies that are specific for losses at the different periods. However, women with repeated preimplantation and occult post-implantation losses will often be diagnosed as infertile or subfertile (**Figure 1**). The findings that women with subfertility

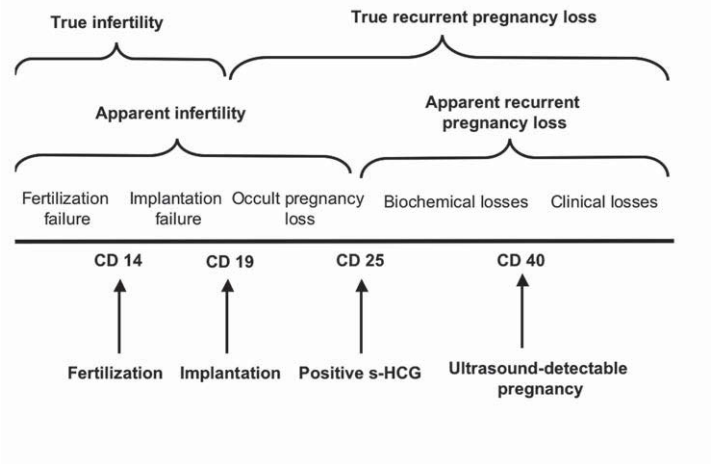

Figure 1. The relationship between time of fertilization, implantation, earliest detection of human chorionic gonadotrophin in serum (s-HCG) and earliest detection of intrauterine pregnancy and apparent and true diagnosis of infertility and recurrent pregnancy loss. CD = cycle day.

have a higher risk of miscarriage (Hakim *et al.*, 1995) and that subfertility is a prognostic negative factor in women with recurrent miscarriage (Cauchi *et al.*, 1995) support the hypothesis that apparent subfertility/infertility and RM have overlapping aetiologies. Indeed, it has been documented that some abnormalities are both associated with implantation failure and miscarriage. Chromosomal abnormalities in the embryo can both negatively affect the implantation potential of preimplantation embryos and cause clinical miscarriage. The presence of autoantibodies is associated with RM, infertility and RIF (Stern *et al.*, 1998; Matsubayashi *et al.*, 2001). In addition, subsets of natural killer (NK) cells (Ntrivalas *et al.* 2001; Michou *et al.*, 2003) and T helper type 1 (Th1) cytokine-producing lymphocytes (Kwak-Kim *et al.*, 2003) in peripheral blood have been reported to be associated with infertility, RIF, sporadic spontaneous abortion and RM.

The general acknowledgement of the hypothesis that losses of conceptions in all stages from preimplantation losses to clinical miscarriages have partly identical aetiologies has caused treatments such as IVF combined with preimplantation genetic screening (PGS), anticoagulation treatment and various immunotherapeutic interventions to be tested in patients with both RIF and RM. It is therefore logical to deal with future directions of research in RIF and RM in a common paper, with focus on research aiming to clarify and treat chromosomal, endocrine, thrombophilic and immunological causes of the syndromes.

Chromosome abnormalities of the conceptus

The most important cause of sporadic miscarriages is fetal aneuploidy, found in 51% of miscarried embryos (Hassold and Chiu, 1985). In older RM patients and RM patients with few miscarriages, the fetal aneuploidy rate is at a similar level (Ogasawara *et al.*, 2000; Stephenson *et al.*, 2002), whereas in RM patients younger than 35 years or with five or more previous miscarriages, the fetal aneuploidy rate is significantly lower (Ogasawara *et al.*, 2000; Stephenson *et al.*, 2002; Rubio *et al.* 2005a,b). A series of studies have reported that embryos (typically from day 3) from women with RM or RIF investigated by PGS exhibit a higher frequency of aneuploidy than embryos from women without these problems. In several publications it has been reported that the rate of chromosomal abnormality was 70.7% in RM (Rubio *et al.*, 2003) and 67.4% in RIF patients (Pehlivan *et al.*, 2003) respectively, which was significantly higher than the abnormality rates of 45.1 (Rubio *et al.*, 2003) or 36.3% (Pehlivan *et al.*, 2003) in embryos from women undergoing PGD for sex-linked diseases. The authors concluded that both RM and RIF patients produced chromosomally abnormal embryos with increased frequency, which might be a pathogenetic factor, and through its potential to select normal embryos for uterine transfer, PGS was proposed as a treatment option for RM and RIF. However, the conclusion that RM and RIF patients produce chromosomally abnormal embryos with increased frequency is dependent on the validity of the results of the control groups, which in the referred studies (Pehlivan *et al.*, 2003; Rubio *et al.* 2003), comprised few patients. Some studies using a similar number of fluorescence in-situ hybridization probes for the PGS procedure have found 45% abnormal blastomeres in embryos from 'normal' women (Bielanska *et al.*, 2002; Ziebe *et al.*, 2003), whereas a very recent study found that as many as 64% of blastomeres of embryos from younger 'normal' IVF patients were abnormal (Baart *et al.*, 2006). Compared with a 64% abnormality rate in controls, RIF and RM women do not appear to produce more aneuploid embryos than other women of comparable age.

Studies of the use of PGS in the treatment of RM and RIF are hampered by the lack of good control groups of adequate size. In one study of patients <37 years with two or more miscarriages (Rubio *et al.*, 2005a,b), the miscarriage rate in pregnancies after PGS was 10.0% compared with 8.3% in a very small control group of 'normal' women. It is difficult to conclude whether there really was an improvement in outcome after PGS, since the spontaneous success rate in younger patients with only two or three miscarriages is >70% (Nybo Andersen *et al.*, 2000) and the subgroup of patients who became pregnant after PGS in the study (approximately 39%) may be patients with a very good spontaneous prognosis with respect to miscarriage rate. A retrospective study using historical controls in women with RIF older than 37 years reported non-significant increases in implantation rate and decreases in miscarriage rate (Platteau *et al.*, 2005), and a non-controlled trial reported a very low miscarriage rate in

RM patients older than 34 years after IVF with PGS (Munné *et al*., 2005). One study has reported that pregnancy rates improve after PGS with RIF under 41 years but not after this age (Taranissi *et al*. 2005), whereas another study found that PGS only benefitted RIF patients of advanced age (over 40 years) if there had been fewer than two previously failed cycles and more than eight zygotes (Munné *et al*. 2003). In a recent review on PGS in RIF, it was concluded that from the published studies there is no firm evidence that patients with RIF will benefit from PGS (Caglar, 2005).

The main reason for the uncertainty of the benefits of PGS in RM and RIF is due to chromosomal mosaicism. Only 9–30% of preimplantation embryos seem to be completely aneuploid, whereas 48–60% are mosaic euploid, aneuploid or polyploid (Bielanska *et al*., 2002; Ziebe *et al*., 2003). Whereas it is reasonable to suggest that embryos with 100% chromosomally abnormal blastomeres will not implant or will miscarry very early, the fate of mosaic chromosomally normal/abnormal embryos is as yet unknown. It has been suggested that cell-cycle checkpoints may not be established in zygotes before the initiation of zygotic gene expression after the 4-cell stage and this is the reason for the high frequency of aneuploid blastomeres in 2–3-day embryos (Delhanty and Handyside, 1995; Harrison *et al*., 2000; Wells *et al*., 2005). When cell cycle control is established, the cleavage of genetically abnormal blastomeres may be arrested and at the blastocyst stage the embryo will in many cases become completely normal. Baart *et al*. (2006) reported that half of day-3 embryos that were found to be mosaic normal/abnormal after biopsies of two blastomeres had become chromosomally normal after developing into blastocysts on day 5. Furthermore, in some cases mosaicism will remain in the trophoblast tissue but the fetus will be completely euploid. Consequently, by discarding embryos with an aneuploid blastomere after PGS, many mosaic embryos that probably would have developed normally are lost and, on the other side, some mosaic normal/abnormal embryos with a euploid blastomere biopsy will be transferred to the uterus but may develop to mainly aneuploid embryos; the risk may be increased after the removal of the normal blastomere (Baart *et al*., 2006). It is extremely important to clarify the potential of PGS to predict how often a 3-day embryo will develop to a normal fetus before this technique is further introduced as a treatment option in women with RIF and RM. At present, it is not known how many diploid cells an embryo needs to be able to develop into a healthy child.

Further longitudinal studies of embryos biopsied on day 3 and subsequent karyotyping of all blastomeres at the blastocyst stage should be undertaken (Baart *et al*., 2006) to clarify the 'cure rate' of mosaic day-3 embryos. More relevant information would come from a study where embryos with abnormal blastomere biopsies are transferred to the uterus by single embryo transfer and prenatal diagnosis by chorion villous biopsy is subsequently undertaken in a subsequent clinical pregnancy, but such a study may never be undertaken for ethical reasons. In RIF, prospective controlled trials must be undertaken to assess the real benefit of IVF with or without PGS. In RM, controlled trials comparing the chance of a live birth in groups matched for age and number of previous miscarriages randomized to treatment with either IVF and PGS or no treatment or standard treatment in a defined period must be undertaken. Whether the application of more complex methods for screening embryonal DNA for abnormalities by PGS will benefit patients with RIF or RM remains to be shown (Urman, 2005b). With regard to PGS and RM, it is important to realize that the spontaneous chance of giving birth declines with the number of miscarriages but the risk of miscarrying a chromosomally abnormal embryo also declines with the number of previous miscarriages (Ogasawara *et al*., 2000). In theory, PGS would be most cost-efficient in RM patients with at least five miscarriages, since the miscarriage rate in placebo-treated patients in this subgroup is as high as 77% (Christiansen *et al*., 2002), but if the rate of aneuploid miscarriages in this group is only between 7% (Christiansen *et al*., 2002) and 28.6% (Ogasawara *et al*., 2000), PGS would at best increase the prognosis between 7/77 = 9% and 28.6/77 = 37% in this group. This is in accordance with the study by Rubio *et al*. (2005a,b), which found that the miscarriage rate per PGS cycle increases with the number of previous miscarriages. In patients with only two or three previous miscarriages, the miscarriage rate after PGS is very low and PGS may be efficient; however, since the miscarriage rate in untreated patients is also very low, it is an open question whether the treatment is cost-efficient in patients with few miscarriages.

Efforts should be made to develop methods that require no blastomere biopsy for selecting embryos with a high implantation chance (and thus may be euploid). Potential useful methods would be computer-controlled multilevel analysis of nuclear structures in 2-cell or 4-cell embryos (Hnida *et al*., 2005) or measurements of soluble human leukocyte antigen-G in IVF culture media (Noci *et al*., 2005). Such methods will be less cost demanding than PGS and the problems of the uncertain predictive value of PGS to detect embryos that will develop to become predominantly aneuploid will be overcome. The increased risk of implantation failure and miscarriage in women of advanced age may be attributed to ageing of the mitochondria in the ooplasm. Preliminary studies suggest that the fertilization and implantation capacity of older eggs may be re-established by transfer of ooplasm or cytoplasm from other cell types in connection with the ICSI procedure (Jansen and Burton, 2004). Large-scale studies with adequate control groups on this topic are awaited.

Endometriosis

It seems that the presence of endometriosis decreases the pregnancy chance (and thus increases the risk of RIF) after IVF (Urman, 2005a). It seems that treatment with gonadotrophin-releasing hormone analogues increase the pregnancy chance after IVF in these patients; however, it remains to be shown in prospective randomized studies whether surgical treatment before IVF is of benefit (Urman, 2005a).

Anatomical abnormalities

A study reported that 26% of RIF patients with normal hysterosalpingograms have intrauterine lesions detected by office hysteroscopy; however, the study provided no documentation that hysteroscopic surgery improves the subsequent implantation rate (Demirol and Gurgan, 2004). Whereas the impact of uterine fibroids and septae in RIF and RM is uncertain and the effect of surgical treatment for these

abnormalities is poorly documented (Urman, 2005a), there is better documentation for a negative impact of hydrosalpinges on implantation and miscarriage rate in IVF patients (Camus *et al.*, 1999; Urman, 2005a). Both the implantation and miscarriage rates seem to be improved after removal of bilateral ultrasound-visible hydrosalpinges (Strandell *et al.*, 1999), but since concerns have been raised whether salpingectomy may compromise ovarian blood circulation (Urman, 2005a) more prospective randomized trials of salpingectomy before IVF are needed.

Endocrine disturbances

The endocrine environment in the ovary and uterus may be important for the chance of implantation and survival of the embryo.

Polycystic ovarian syndrome

Many studies have shown that women with polycystic ovarian syndrome (PCOS) exhibit an increased rate of miscarriage and probably also a decreased rate of implantation. However, most PCOS patients are obese and obesity *per se* seems to increase the miscarriage rate independently of the presence of PCOS. In a multivariate analysis of pregnancy outcome in large cohorts of women undergoing assisted reproduction, high body mass index (BMI) had a highly significant impact on subsequent miscarriage rate, whereas PCOS had no impact after adjusting for other risk factors such as BMI (Fedorcsak *et al.*, 2000; Wang *et al.*, 2002a). Endocrine, anti-fibrinolytic and metabolic abnormalities associated both with PCOS and obesity: high follicular phase LH concentrations, hyperandrogenaemia, hyperinsulinaemic insulin resistance, high plasminogen activator inhibitor activity, elevated leptin concentrations or high oestradiol concentrations on the day of HCG in IVF cycles have been proposed as factors causing the fecundity problems in PCOS by affecting the oocyte quality or endometrial maturation and receptivity. With regard to the latter factor, a study found that high oestradiol concentrations on the day of oocyte retrieval in IVF cycles were associated with an increased miscarriage rate after adjustment for age and BMI (Wang *et al.*, 2002a), but a review of relevant studies could not detect any association between oestradiol concentrations on the day of HCG administration and pregnancy achievement in IVF cycles (Kosmas *et al.*, 2004). Two major questions remain unsolved: (i) what is (are) the factor(s) directly causing decreased implantation rate and increased miscarriage rates in obese women and (ii) is (are) the factor(s) exerting its/their effect by diminishing embryo quality or by decreasing endometrial receptivity?

To clarify the role of the obesity-associated serological abnormalities in infertility and miscarriage prospective studies of conception rates and pregnancy outcome should be undertaken in large groups of untreated women using multivariate analyses adjusting for the interaction of the aforementioned serological factors, age, BMI, waist circumference, duration of infertility, previous pregnancy losses or births and ovarian ultrasonographic morphology.

To distinguish between the contribution of obesity-related serological disturbances on the oocytes and the endometrium with respect to the implantation and miscarriage rates, studies of women undergoing oocyte donation are extremely informative, since any difference in implantation and miscarriage rate between lean and obese women must be explained by differences in endometrial receptivity or effects of the serological factors on the post-implantation embryo. So far, only one such study has been carried out showing that embryo recipients with high BMI exhibited a much higher miscarriage rate that recipients with normal BMI (Bellver *et al.*, 2003). More studies of the same kind should be done and when more is known about the effects of PCOS-/obesity-related factors on oocyte and endometrial quality respectively, it will be possible to design treatment studies optimally with respect to type and duration of medication.

Relevant studies would be placebo-controlled trials of pre-conceptional contraceptive pill usage, long-term GnRH-analogue or GnRH-antagonist treatment, metformin treatment and combinations of these using different stimulation protocols. Prospective trials should also be performed where randomization between ovarian diathermy and no diathermy is undertaken in PCOS patients.

LH concentrations

Hypersecretion of LH (plasma follicular phase concentrations >10 IU/l) can be found in 8% of RM patients (Li *et al.*, 2000), but neither LH nor testosterone concentrations were predictive of miscarriage in RM patients (Nardo *et al.*, 2002) and suppression of LH with GnRH analogues did not affect the miscarriage risk (Clifford *et al.*, 1996). In accordance with these studies are the aforementioned multivariate analyses (Fedorcsak *et al.*, 2000; Wang *et al.*, 2002a) showing that obesity *per se* and not endocrine factors such as LH and androgens is the main risk factor for miscarriage in patients with PCOS.

Low LH concentrations in the mid-follicular phase have also been reported to be associated with significantly increased miscarriage rates in assisted reproduction cycles using GnRH agonist down-regulation and ovarian stimulation with recombinant FSH (Westergaard *et al.*, 2000). A meta-analysis of controlled trials comparing human menopausal gonadotrophin (HMG) with recombinant FSH ovarian hyperstimulation after GnRH agonist down-regulation showed that HMG stimulation resulted in significantly higher clinical pregnancy rates, but not decreased miscarriage rates (van Wely *et al.*, 2003). The higher clinical pregnancy rates may be attributed to fewer patients with highly suppressed LH concentrations in the mid-follicular phase in the HMG group. Controlled trials comparing stimulation protocols associated with low mid-follicular LH concentrations with protocols associated with higher LH concentrations should be undertaken in women with RIF or RM after IVF with documented low mid-follicular LH concentrations during previous treatment cycles.

Luteal phase defects

Inadequate secretion of progesterone by the corpus luteum in the luteal phase and in the early weeks of pregnancy has traditionally been considered a causative factor in many cases of infertility, miscarriage and RM. In one study using delayed maturation of repeated late luteal phase endometrial

biopsies as a criterion for luteal phase deficiency, 34 (17.3%) of 197 patients with RM were diagnosed as having the disorder (Stephenson, 1996). A meta-analysis of all relevant placebo-controlled trials of progestogen treatment for preventing miscarriage could not detect any effect at all, however, in a subset of 93 patients with RM a significant treatment effect of progestogens was found (Oates-Whitebread *et al*., 2005). These patients had entered three small trials from before 1964, some of which had poor allocation concealment, a substantial number of post-randomization exclusions or inclusion late in the first trimester. Given the trend for a lower miscarriage rate in RM patients in this meta-analysis, recent evidence that progestagens can switch cytokine production in lymphocytes from RM women towards production of Th2 cytokines that are probably beneficial for pregnancy (Raghupathy *et al*., 2005) and results suggesting a role of progesterone in preventing preterm birth (Meis *et al*., 2003), the need for more placebo-controlled trials of progesterone treatment of RM patients is emphasized. Such trials should be of adequate size, test natural progesterone and treatment should start as soon as the HCG test is positive at the time of the missed period.

Thrombophilia

A series of acquired and congenital non-antiphospholipid (APL) factors causing an increased clotting tendency of the blood have in some studies been found with higher prevalence in RM women than in relevant controls, however the association is strongest (Rey *et al*., 2003) or may only be present in patients with second trimester miscarriages (Roque *et al*., 2004). Prospective studies regarding the impact of the most prevalent thrombophilia factor, the factor V Leiden mutation, on the miscarriage rate have provided very contradictory results, with some studies showing that carriers of the mutation have an increased (Rai *et al*., 2002) and other studies (Carp *et al*., 2002; van Dunne *et al*., 2005) a decreased rate of first trimester miscarriage. The frequency of non-APL thrombophilia factors has been reported to be significantly increased in women with unexplained infertility or RIF after IVF (Azem *et al*., 2004). Prospective studies of the impact of the factor V Leiden mutation have reported that its presence increases (Göpel *et al*., 2001) or does not affect (van Dunne *et al*., 2005) the chance of conception. It is thus so far very uncertain whether non-APL thrombophilia factors cause first trimester RM or RIF. Before this is clarified in more prospective studies of large cohorts of otherwise comparable RM or RIF patients positive and negative for these factors, treatment of positive patients with anticoagulation inside or outside placebo-controlled trials is premature and should not be undertaken.

APL are autoantibodies that in some cases can give rise to a thrombophilic condition especially if lupus anticoagulant activity is present. These antibodies will also be discussed in the section on autoantibodies. APL have in many studies been found with increased prevalence in RM women and in some studies also in RIF patients. The majority of studies have reported that APL antibodies affect the future miscarriage rate negatively (Nielsen and Christiansen, 2005), whereas they do not seem to affect the implantation rate in assisted reproduction (Eldar-Geva *et al*., 1999; Chilcott *et al*., 2000; Stern *et al*., 2003). Only three small non-blinded controlled trials of heparin/low-dose aspirin versus low-dose aspirin have been undertaken in RM patients, with contradictory results (Kutteh 1996; Rai *et al*., 1997; Farquharson *et al*., 2002). There is obviously a need for more and larger controlled trials of heparin/aspirin versus no treatment (or preferably placebo) in APL-positive RM women (Lassere and Empson, 2004). Concerning heparin/aspirin treatment of women with APL and RIF, Stern *et al*. (2003) found no effect of the treatment in a placebo-controlled trial.

Immunology

Cytokines

During human pregnancy a semi-allogeneic fetus implants in the uterus, and at the feto–maternal interface a maternal immune reaction directed against alloantigens on the fetus or trophoblast is expected to take place. Cytokines are important mediators of signals between cells of the immune system and other cells. For some years, a prevalent theory has been that predominant production of so-called T helper type 2 (Th2) cytokines such as interleukin (IL)-4 and IL-10 was characteristic of normal implantation and pregnancy, whereas in miscarriage and RM there was a predominant production of Th1 cytokines such as interferon (IFN)γ and IL-2 (Wegmann *et al*., 1993). Th2 cytokines are known to direct the immune reaction towards a humoral response, which may not harm the feto–placental unit, whereas Th1 cytokines direct the reaction towards a cytotoxic response or the cytokines may be harmful *per se*.

Animal studies

Much information about the role of immunological processes in implantation failure and pregnancy loss has been derived from studies of knock-out mice. The classical study was undertaken in leukaemia inhibitory factor (LIF) knock-out mice, finding a crucial role for maternal LIF in implantation (Steward *et al*., 1992). In support of the Th1/Th2 theory it was found that IL-10 knock-out mice when given very low doses of lipopolysaccharide (LPS) display a high fetal resorption rate associated with a significant increase in NK cell activation and invasion, which could both be reversed by administration of IL-10 or neutralization of tumour necrosis factor (TNF)α (Murphy *et al*., 2005). It was concluded that inflammatory stimuli in the uterus activate uterine NK cells to secrete TNFα that harms the fetuses or the placenta when IL-10 is not present. In opposition to the Th1/Th2 theory is a study showing that the production of the typical Th1 cytokine IFNγ by uterine NK cells seems to be a pre-requisite for normal fetal implantation in mice (Ashkar and Croy, 2001) Although probably not directly affecting cytokine production, indoleamine 2,3 dioxygenase (IDO) may play a crucial role for pregnancy success, at least in mice since blockage of IDO results in loss of allogeneic but not syngeneic concepti a few days after implantation (Munn *et al*., 1998). This was caused by a massive inflammatory reaction with complement activation at the feto–maternal interface driven by T-cell recognition of fetal antigens.

Human studies

Several studies of the role of various cytokines in implantation and pregnancy have been undertaken in humans. In-vitro studies have shown that Th1 cytokines such as TNFα and IFNγ inhibit

growth and induce apoptosis of human trophoblast cells (Yie *et al.*, 1994; Ho *et al.*, 1999).

To elucidate what is the cause and what is the effect, reliable studies of immunological factors and miscarriage in humans should be done before pregnancy or in very early pregnancy several weeks before fetal death may occur (Hill *et al.*, 1995; Lachapelle *et al.*, 1996; Kruse *et al.*, 2003), but at the time of pregnancy loss, studies are only valid if the fetal karyotype is known (Yamamoto *et al.*, 1999; Quack *et al.*, 2001; Yamada *et al.*, 2001) because changes in immune parameters in relation to miscarriage of a karyotypically abnormal embryo must be considered as secondary to the inflammatory response against necrotic tissue retained in the uterus.

Studies of cytokines prior to pregnancy

Lower expression of IL-1β and IL-6 mRNA (von Wolff *et al.*, 2000) and LIF and IL-6 protein (Laird *et al.*, 2003) has been found in endometrial biopsies from non-pregnant RM patients than in biopsies from normal women. Strong expression of IL-12 or an almost absence of IL-12 and IL-18 expression in endometrial biopsies taken in the luteal phase was found in 75% of patients with RIF but in none of parous controls (Ledee-Bataille *et al.*, 2004a). However, in IVF patients detectable concentrations of IL-18 in uterine flushings from the day of oocyte retrieval reduced the subsequent implantation rate and pregnancy rate significantly compared with the absence of IL-18 (Ledee-Bataille *et al.*, 2004b). IL-18 alone is thought to be a Th2-promoting cytokine whereas IL-12 in high concentrations is a Th1-promoting cytokine. Another study found that both women with RM and women with RIF after IVF had significantly higher ratios of TNFα/IL-4- and TNFα/IL-10-producing T helper lymphocytes in peripheral blood before pregnancy or IVF treatment compared with multiparous women (Kwak-Kim *et al.*, 2003). Hill *et al.* (1995) reported that peripheral blood lymphocytes from non-pregnant RM women after stimulation with trophoblast antigens produced IFNγ much more often than lymphocytes from normal parous control women.

Studies of cytokines during pregnancy

Kruse *et al.* (2003) found that RM women who subsequently miscarried had a significantly higher production of TNFα from peripheral blood lymphocytes collected from the very early stages of pregnancy compared with those who subsequently gave birth. In RM women, peripheral lymphocytes can be demonstrated to produce increased concentrations of mediators of inflammation IFNγ and TNFα before miscarriage happens (Mueller-Eckhardt *et al.*, 1994). However, production of Th type 1 cytokines and TNFα in early pregnancy was more characteristic for women with normal pregnancies than for RM patients in a study by Bates *et al.* (2002).

Cytokine gene polymorphisms

Instead of measuring cytokines or cytokine production directly, many studies have investigated the frequency of polymorphisms of cytokine genes in RIF and RM patients and relevant controls. Daher *et al.* (2003) published a meta-analysis of studies of the association between cytokine gene polymorphisms and RM. It showed that high cytokine production genotypes of IFNγ (+874 T/T) and IL-10 promoter (–1082 G/G) were significantly increased in RM patients and there was a non-significant trend for an increased prevalence of high-production TNFα promoter –308 A/A and A/G genotypes. Prigoshin *et al.* (2004) later reported a significant increase in the IFNγ intermediate-production genotype +874 T/A in RM women and a small but significant increase in the TNFα –308 A/A and G/A genotypes supporting the findings in the meta-analysis. However, Pietrowski *et al.* (2004) found no association between two polymorphisms in the TNFα promoter alleles, one of which was the –308 polymorphism, and RM and Costeas *et al.* (2004) found no differences between abortion-prone women and fertile controls with regard to the prevalence of the previously mentioned IL-6 promoter, IFNγ intron 1 +874, TNFα promoter and IL-10 promoter polymorphisms. Wang *et al.* (2002b) found that female carriage of the variants IL-1B-511*1 and IL-1B-31*2 were associated to Th1 immunity and RM and Perni *et al.* (2004) found that fetal carriage of homozygosity for the IL-1 receptor antagonist allele 1 was associated with reduced intra-amniotic IL-1β concentrations and an increased occurrence of miscarriage in previous pregnancies. However, others could find neither IL-1B-511*1 nor other IL-1B polymorphisms with any changed frequencies in large studies of RM patients and controls (Hefler *et al.*, 2001, 2002; Linjawi *et al.*, 2005). It is thus at the moment unclear whether there is any relationship between cytokine gene polymorphisms and RIF and RM. So far, the most convincing association is between polymorphism in the IFNγ gene and RM but larger case–control studies and prospective studies are needed both in RIF and RM patients.

The majority of studies in animals and humans seem to support the theory that too high production of Th1 cytokines locally or systemically is associated with RIF and RM, but that some production of Th1 cytokines, including TNFα, may be a requisite for normal implantation and very early pregnancy. The Th1/Th2 hypothesis is probably too simplistic and hypotheses are now propagated that to ensure a successful outcome in pregnancy from implantation to birth, some degree of uterine or systemic inflammation mediated by inflammatory cytokines such as IFNγ and TNFα is necessary but if inflammation becomes too weak or too strong pregnancy complications such as miscarriage, intrauterine growth retardation and pre-eclampsia may occur (Redman and Sargent, 2003).

NK cells

Animal studies

Uterine and peripheral blood NK cells are interesting in the context of RIF and RM since $CD56^+$ NK cells constitute the major lymphocyte population in the decidua. Guimond *et al.* (1998) found that NK-deficient mice display abnormalities in decidual artery remodelling and trophoblast invasion, probably due to lack of uterine NK cell derived IFNγ, and the mice display an increased rate of fetal growth retardation, but reconstituting the NK cells by bone marrow transplantation normalizes the abnormalities. The previously mentioned study by Ashkar and Croy (2001) also pointed towards a role for uterine NK cells in fetal resorptions in mice.

Human studies

A study of biopsies from the endometrium in non-pregnant patients with RM and fertile controls showed that RM women harbour a much higher frequency of $CD16^{+}CD56^{dim}$ NK cells (expressing low concentrations of the CD56 marker) and a lower frequency of $CD16^{-}CD56^{bright}$ NK cells (exhibiting a higher CD56 surface density expression) than the controls (Lachapelle *et al.*, 1996). Ntrivalas (2001) found that an increase in $CD16^{+}CD56^{dim}CD69^{+}$ NK cells in peripheral blood was characteristic of women with RM and unexplained infertility, and Thum *et al.* (2004) found that the absolute count of the same NK cell subset in IVF patients was associated with a low implantation rate and high miscarriage rate compared with a low absolute count. CD69 is an activation marker on NK cells. A prospective study of peripheral blood NK cell cytotoxicity at 2-week intervals during early pregnancy in RM women showed that the toxicity decreased significantly in women who later gave birth or miscarried a chromosomally abnormal fetus, whereas women who miscarried chromosomally normal fetuses exhibited a significantly increased NK cell activity in the same period (Yamada *et al.*, 2001). In other studies, a significantly lower percentage of $CD56^{+}$ decidual NK cells were found in chromosome-normal compared with chromosome-abnormal missed abortions and induced abortions (Yamamoto *et al.*, 1999; Quack *et al.*, 2001). A lack of $CD56^{bright}$ NK cells in the decidua thus seems to predispose to chromosomally normal miscarriage and RM. The increased peripheral blood NK cytotoxicity and decreased percentage of decidual $CD56^{bright}$ NK cells in chromosomally normal miscarriage may reflect an aberrant (maybe to low) inflammatory response in this kind of miscarriage because $CD56^{bright}$ NK cells have been shown to produce significantly higher concentrations of IFNγ, TNFβ and IL-10 (and also TNFα) in response to the monokines (soluble mediators of immune responses that are produced by monocytes or macrophages) IL-12, IL-15 and IL-18 compared with $CD56^{dim}$ cells (Cooper *et al.*, 2001). Instead, $CD56^{dim}$ cells seem to exhibit a higher NK cytotoxic activity. Overall, these studies have shown that uterine $CD56^{bright}$ NK cells are necessary for early normal pregnancy, probably by secreting both pro- and anti-inflammatory cytokines the relative proportions of which determine the degree of inflammation at the feto–maternal interface, which must be within some intervals.

Autoantibodies

There is broad agreement that a series of autoantibodies, especially antiphospholipid antibodies and antinuclear antibodies, can be found with increased prevalence in RM women and they also display a prognostically negative impact (Nielsen and Christiansen, 2005). The majority of studies have also found an increased prevalence of these autoantibodies among IVF and RIF patients (Birkenfeld *et al.*, 1994; Coulam *et al.*, 1997; Stern *et al.*, 1998; Chilcott *et al.*, 2000; Matsubayashi *et al.*, 2001); however, prospective studies in untreated (Chilcott *et al.*, 2000; Kikuchi *et al.*, 2003) and anticoagulation treated (Stern *et al.*, 2003) patients have concluded that the presence of these antibodies exhibits no negative impact on outcome in IVF patients and heparin/aspirin treatment of antiphospholipid antibody positive RIF patients has no documented effect (Urman, 2005b).

These autoantibodies may in some instances have developed as a result of supraphysiological oestrogen concentrations in previous IVF/ICSI cycles or they may have developed as a consequence of previous ovarian punctures (Gobert *et al.*, 1992; Birkenfeldt *et al.*, 1994). Clearly, longitudinal studies of autoantibody concentrations in infertile women before starting IVF/ICSI and after each IVF/ICSI cycle are warranted.

Immunogenetics

Studies of genetic factors of importance for the immune function in women with miscarriage or RM compared with normal controls can potentially provide information as to whether the former patients carry particular genetic determinants more often than relevant controls. Of particular relevance are studies of polymorphic promoter regions and alleles of cytokine genes, polymorphic genes coding for complement-related factors and alleles of the major histocompatibility antigens (HLA). The cytokine gene polymorphisms have been discussed previously.

Mannose-binding lectin

Mannose-binding lectin (MBL) is an important constituent of the innate immune system and is also one of the many proteins of the complement system providing a third pathway of complement activation in addition to the classical and alternative pathways. In addition, MBL has been proposed to display anti-inflammatory properties by modulating the intracellular cytokine production by monocytes and by suppressing the production of the three inflammatory cytokines TNFα, IL-6 and IL-1β (Jack *et al.*, 2001). It has also been found that MBL binds to apoptotic cells thereby stimulating their ingestion by phagocytes (Ogden *et al.*, 2001). This may prevent and resolve inflammation at the feto–maternal interface, since trophoblast cell debris has been suggested to be an important stimulus for NK-cell-mediated inflammatory reactions in pathologic pregnancy (Borzychowski *et al.*, 2005).

Low concentrations of MBL are associated with RM, and women with low MBL concentration exhibit a diminished pregnancy prognosis (Kilpatrick *et al.*, 1995; Kruse *et al.*, 2002); however, it has not been possible to detect any association between low MBL and implantation failure or infertility (Hartwell *et al.*, abstract 0–171, 21st Annual Meeting of ESHRE, 2005)

HLA

In particular, one HLA loci has been extensively studied in RIF and RM: HLA-G. The non-classical HLA molecule HLA-G exhibits a limited tissue distribution and a low polymorphism. It has attracted much attention, since it is one of the few HLA antigens that is expressed on invasive trophoblast cells. Membrane-bound HLA-G *in vitro* strongly reduces IFNγ production of unfractionated uterine mononuclear cells but stimulates cytokine production by purified uterine NK cells (van der Meer *et al.*, 2004). *In vitro*, HLA-G expression on target cells may stimulate peripheral blood mononuclear cells to secrete the anti-inflammatory cytokine IL-10 and inhibit the secretion of TNFα and IFNγ (Maejima *et al.*, 1997; Kapasi *et al.*, 2000) and failure to express HLA-G is thus expected to result in a harmful inflammatory response.

Absence of soluble HLA-G (s-HLA-G) in the IVF culture media has been reported to be strongly associated with a low implantation rate (Sher *et al.*, 2004; Noci *et al.*, 2005; Yie *et al.*, 2005) and tests

for HLA-G secretion are promising candidates as useful methods for identifying good embryos for uterine transfer.

In addition to trophoblast, s-HLA-G is also produced by monocytes and can be detected, although in low concentrations in the sera of non-pregnant women and men. Low s-HLA-G concentration in serum has been reported as being predictive for subsequent miscarriage in both non-pregnant and pregnant IVF patients (Pfeiffer *et al*., 2000).

A study has shown that some HLA-G alleles (linked to a 14 base pair insertion in exon 8 of the HLA-G gene) are associated with a low production of soluble HLA-G, and these alleles are found with increased prevalence in RM patients and women with repeated implantation failure after IVF (Hviid *et al*., 2004a,b). This supports the hypothesis that s-HLA-G may play a positive role for trophoblast invasion and growth from the implantation stage until the end of the first trimester. The 14 base pair insertion is in linkage disequilibrium with HLA-DR3 (Hviid and Christiansen, 2005), an HLA class II allele found with a highly significantly increased prevalence in women with RM compared with controls (Kruse *et al*., 2004). The association between HLA-DR3 and RM may have two explanations: lymphocytes from HLA-DR3 positive individuals *in vitro* are high-secretors of TNFα (Pociot *et al*., 1993) and low soluble HLA-G concentrations are associated with HLA-DR3 due to linkage disequilibrium with HLA-G polymorphisms. Both features predispose to increased inflammation at the feto–maternal interface and miscarriage.

Immunological treatments

Glucocorticoid (prednisone) treatment inhibits production of a series of inflammatory cytokines but in a large, placebo-controlled study it did not significantly improve live birth rate significantly in RM patients with evidence of autoimmunity treated during all of pregnancy with high doses (Laskin *et al*., 1997). Its use was associated with a series of maternal and fetal side effects in late pregnancy. However, the trial showed a trend for a lower miscarriage rate in the prednisone-treated compared with placebo-treated patients (65 versus 56%) and other studies have suggested that lower doses of prednisone may improve the live-birth rate after IVF (Ando *et al*., 1996; Hasegawa *et al*., 1998; Geva *et al*., 2000) and can normalize NK cell subpopulations in the endometrium of RM patients (Quenby *et al*., 2005). Therefore, trials of prednisone treatment in RIF and RM patients where prednisone is administered only before conception and in early pregnancy are warranted.

Allogeneic lymphocyte immunization using the partner's or third party lymphocytes has been reported to be efficient especially in patients with primary RM (Daya and Gunby, 1994); however, the current Cochrane meta-analysis on immunotherapy in RM (Scott, 2003) does not make separate analysis in patients with primary RM and concludes there is no effect of the treatment, although the odds ratio for live-birth in patients treated with third party lymphocytes was 1.39 (95% confidence intervals 0.68–2.82) in the combined group of primary/secondary RM patients. In primary RM it has been shown to exhibit a significant treatment effect compared with placebo (Christiansen *et al*., 1994). Further placebo-controlled trials of third party lymphocyte immunization in RM are needed.

Intravenous immunoglobulin (i.v. Ig) modulates cytokine production in addition to many other immune modulating effects. Placebo-controlled trials using adequate doses of i.v. Ig have so-far suggested that it has a significant therapeutic effect in women with secondary RM and women with repeated second trimester RM, whereas the effect in patients with primary RM seems to be minimal (Christiansen *et al*., 2002). However, most trials have used i.v. Ig doses much lower than those normally used in autoimmune disorders, in many studies the treatment has started so late in pregnancy that the drug had not been given enough time to exert its effects and in several studies only patients with primary RM have been included. The Cochrane meta-analysis of immunotherapeutic trials in RM has not made any distinction between trials with adequate i.v. Ig administration and has not done separate analysis of the effect in patients with primary and secondary RM (Scott, 2003). Therefore the analysis concludes that there is no evidence for a treatment effect of i.v. Ig whereas another meta-analysis focusing on patients with secondary RM found that the odds ratio for live births in the i.v. Ig treated patients was 1.60 (95% confidence intervals 0.70–3.66), which was almost significant (Christiansen and Nielsen, 2005). More placebo-controlled trials of i.v. Ig using adequate doses starting from very early pregnancy (or from before conception) must be undertaken to clarify the effect of i.v. Ig on miscarriage rate and relevant immunological parameters in RM women.

Three trials of i.v. Ig in patients with repeated IVF failures have been published. One placebo-controlled trial could not find any significant improvement in live birth rate in cycles where i.v. Ig was administered compared with placebo (Stephenson and Fluker, 2000), whereas two other trials without untreated controls found astonishing high live-birth rates in i.v. Ig treated patients with repeated IVF failure and various immunological disturbances (Coulam and Goodman, 2000; Scher and Salazar, 2000). The encouraging results from these two studies and the experience derived from the studies of i.v. Ig in RM warrant the performance of more prospective placebo-controlled trials in patients with RIF after IVF.

Methodological issues of research in RIF and RM

As reviewed in a recent paper (Christiansen *et al*., 2005) the majority of studies in the area of RM and probably also RIF are subjected to serious methodological flaws, which in many cases invalidate the results. The most prevalent flaws are inclusion of patients with only two previous miscarriages, ascertainment bias (a selection bias happening when patients are referred to clinics or studies with increased chance because of a particular clinical or paraclinical feature), use of inappropriate controls and late inclusion in treatment trials.

As mentioned previously, a common flaw in immunological research is to conclude that some findings that may be caused by miscarriage are causing miscarriage. The present review has therefore aimed at referring to studies without serious methodological flaws. It is of extreme importance to minimize methodological flaws in future research in RIF and RM: this can be done by educating scientists in identifying and avoiding methodological flaws but reviewers and editors of journals also bear a responsibility for improving the methodological quality of published papers.

Conclusions

The research problems that most urgently need to be solved within each category of potential causes of RIF and RM are listed in **Table 1**. When these questions can be adequately answered, the final goal should be achieved: to significantly diminish the number of patients who have to terminate IVF/ICSI treatment or treatment for RM without having achieved a successful pregnancy.

It is important to realize that most patients will have more than one potential cause after having passed an extensive investigation programme (Christiansen *et al*., 2005), and it is thus important not to cancel the remaining investigations once one potential cause is found both in research protocols and clinical practice. Probably each identified risk factor adds to the patient's total risk of RIF or RM and recognition of this fact is necessary prerequisite for designing research trials that can adequately answer the questions being posed: e.g. in placebo-controlled trials of treatment, the prevalence of the most important risk factors for RIF and RM should be equally distributed in the two allocation groups.

To carry out better and larger studies in the field of RIF (and RM) collaboration between many laboratories and clinical units is needed in order to collect the necessary expertise and number of patients. Such collaboration requires money, and this has fortunately been recognized by the European Union that recently allocated economic funding for establishing collaboration between a large number of European laboratories and IVF units in order to promote high-quality research in failed implantation. There are good reasons to believe that this European Network of Excellence entitled EMBIC (Embryo Implantation Control) will be able to solve at least some of the questions posed in **Table 1** in the future.

Table 1. Important questions that need to be answered in recurrent implantation failure (RIF) and recurrent miscarriage (RM) research.

Research area	*Research question*
Genetics	How often will mosaic embryos biopsied on day 3 develop to a normal fetus? What is the benefit of IVF with PGS in young and elderly patients, respectively, with RIF and RM in a cost–effectiveness analysis? Can reliable and quick methods be developed for identifying embryos with a high implantation potential that require no blastomere biopsy?
Endocrinology	What is the impact of the obesity-related serological abnormalities in infertility and miscarriage in prospective studies after adjustment for the interaction between the serological factors, age, BMI, waist circumference, duration of infertility, previous pregnancy losses or births and ovarian ultrasonographic morphology? What is the impact of the obesity-related serological disturbances on the implantation and miscarriage rates in women undergoing oocyte donation? Can treatment of RM patients with natural progesterone starting as soon as the HCG test is positive be proved to be effective in up-to-date placebo-controlled trials of adequate size? Can treatments with drugs correcting obesity-/PCOS-related serological factors be proved to be effective in increasing implantation or decreasing miscarriage rates in placebo-controlled trials of obese/PCOS patients?
Thrombophilia	Are there any differences in implantation rate or pregnancy outcome between large cohorts of untreated RIF and RM patients positive and negative for non-APL thrombophilia factors but otherwise comparable? Do heparin/aspirin improve the pregnancy prognosis compared with no treatment (or preferably placebo) in APL-positive RM women?
Immunology	What is the relationship between relevant measures of immune function (cytokine production, NK- and T-cell function) in the uterus and the peripheral blood during human pregnancy? What are the mean values and confidence limits of relevant immune parameters in the uterus and peripheral blood in each gestational week during the first trimester of human pregnancies that are electively terminated or miscarry in spite of the embryo being chromosomally normal? What is the relationship between maternal and fetal HLA-G and HLA-DR–DQ alleles, soluble HLA-G secretion and RIF and RM in case–control and prospective studies of adequate size using up-to date laboratory methods? Will prednisone used only before conception and in early pregnancy increase the implantation rate and the live birth rate in RIF patients and RM patients respectively with acceptable side effects? Will i.v. Ig increase the live-birth rate in patients with secondary RM in placebo-controlled trials using adequate doses and early initiation of the infusions?

APL = anti-phospholipid; BMI = body mass index; HCG = human chorionic gonadotrophin; HLA = human leukocyte antigen; Ig = immunoglobulin; NK = natural killer; PCOS = polycystic ovarian syndrome; PGS = preimplantation genetic screening.

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