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ORIGINAL ARTICLE *Early pregnancy*

Recurrent pre-clinical pregnancy loss is unlikely to be a 'cause' of unexplained infertility

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BACKGROUND: A proportion of women with 'unexplained' infertility may present with subfertility because their pregnancies fail before they are clinically recognized. In order to test whether pre-clinical early pregnancy losses (EPL) occur more frequently in women with unexplained infertility, serial urinary hCG concentrations were measured to compare EPL per cycle rates following spontaneous conception in patients with unexplained infertility versus healthy volunteers.

METHODS: Sixty patients under 39 years of age with unexplained infertility and 60 healthy controls, who were trying to conceive spontaneously, participated in this study. All participants were asked to collect daily urine samples from cycle day 14 until menstruation for three consecutive cycles or until a positive pregnancy test was obtained.

Urinary hCG and creatinine levels were measured by immunoassay. Implantation was detected when urinary hCG levels rose above reference levels constructed from samples obtained from 12 women not attempting to conceive. EPL rates were determined by a linear mixed model using logarithmically transformed hCG/creatinine data.

RESULTS: In the 133 cycles of 60 women with unexplained infertility, just one implantation was detected, which became an ongoing pregnancy. In contrast, in 103 such cycles in 46 control patients, 30 implantations were detected (24 clinical pregnancies, 6 cases of EPL). The odds ratio for EPL/cycle in the unexplained versus control group was 0 (95% confidence interval: 0–0.795, $P = 0.026$).

CONCLUSIONS: Our data do not support the hypothesis that recurrent EPL may present as unexplained infertility. Post-implantation failure is therefore unlikely to contribute significantly to the presentation of subfertility.

Key words: pre-clinical early pregnancy loss / hCG / implantation / unexplained infertility

Introduction

'Unexplained' infertility indicates the absence of a definable cause for the failure of a couple to achieve pregnancy after 12 months of striving for conception, despite a thorough evaluation (Moghissi and Wallach, 1983) and accounts for 15–25% of couples presenting with infertility (Templeton and Penney, 1982; Smith *et al.*, 2003). Many putative causes of unexplained infertility have been proposed (Pandian *et al.*, 2005); these include cervical factors, problems with sperm and egg transport or interaction (fertilization failure), failure of the embryo to develop and failure of implantation. In addition, since post-implantation pre-clinical pregnancy loss is known to frequently occur following spontaneous conception (Macklon *et al.*, 2002), it can be proposed that an increased frequency of post-implantation losses in certain individuals may present as 'unexplained' infertility. Clinical subfertility could therefore arise as a result of a malfunction of the endometrial-embryo 'dialogue' after the early phases of implantation. In this case, the occurrence of normal menstrual periods would mask pre-clinical, early pregnancy losses (EPL), which could be mistakenly interpreted as a failure to conceive.

The development of sensitive and specific immunoassays for the detection of urinary hCG has made it possible to detect a pregnancy within a few days of embryo implantation (Wilcox *et al.*, 1985; Canfield *et al.*, 1987; Macklon *et al.*, 2002). In a classic study of 221 healthy volunteers who were trying to conceive, the patterns of hCG rise and decline were measured in serial urine samples in

order to detect the incidence of ongoing pregnancy and EPL, 31% of all conceptions failed, two-thirds of which occurred before clinical detection (Wilcox *et al.*, 1988). Similar high rates of post-implantation, pre-clinical pregnancy loss have been reported by others (Chard, 1991; Zinaman *et al.*, 1996; Macklon *et al.*, 2002; Wang *et al.*, 2003).

The incidence of EPL in subfertile populations remains unclear, as previous studies have generated contradictory results. A positive relationship between the occurrence of an EPL and the chance of achieving a live birth in a subsequent pregnancy has been observed in presumed fertile populations (Wilcox *et al.*, 1988; Wang *et al.*, 2003), in a small study of subfertile women (Sharp *et al.*, 1986) and following conception by IVF (Barlow *et al.*, 1988).

A higher incidence of EPL associated with subfertility was demonstrated in one study in which healthy volunteers with a variety of fertility histories (including impaired fertility), participated (Hakim *et al.*, 1995): in the impaired fertility group, a rate of early pregnancy loss of 70% was observed, compared with 21% in women without fertility problems.

In couples with the specific diagnosis of 'unexplained' infertility, the rate of EPL is unknown. In order to discern whether unexplained infertility can be partly attributed to pre-clinical pregnancy loss, we employed serial urinary measurements of hCG to identify implantation and subsequent EPL or ongoing pregnancy in a large cohort of patients with unexplained infertility and a control population consisting of healthy (presumed fertile) volunteers.

Materials and Methods

Subjects

Ethical approval for the study was obtained from the local institutional Committee, and written informed consent was obtained from all study participants.

Between August 2006 and April 2008, all women under 39 years of age with primary or secondary unexplained infertility of at least 1.5 years duration attending the University Medical Centre Utrecht (The Netherlands) for IVF treatment were invited to participate in this prospective cohort study. Criteria for the diagnosis of infertility of unexplained origin were the presence of a regular ovulatory cycle, a normal result in a test of tubal patency (either hysterosalpingogram/laparascopy or negative chlamydia serology in women with no other risk factors for tubal disease), basal FSH levels in the normal range and normal semen analysis in their partner, according to World Health Organization Criteria (World Health Organization, 1999). All women in the study group had undergone at least three unsuccessful IUI treatments before presenting in our Clinic. The control group consisted of volunteers recruited from an internet 'chat site'. Control subjects were required to have a regular menstrual cycle and no known fertility problems, and had stopped birth control methods a maximum of 3 months before enrolment the study.

All participants were asked to freeze first early morning voiding urine samples taken daily from cycle day 14 until menstruation or a positive pregnancy test in order to detect an embryo implantation. Three cycles were analyzed intentionally. Urine samples were frozen in home freezers (-20°C), until collection and remained frozen until analysis.

Low levels of hCG are present in normal human cyclic endometrium in the secretory phase (McChesney *et al.*, 2005), which may reflect a function of hCG in the differentiation of human endometrial cells into decidua (Delbaere, 2008). In order to produce a reference curve of the luteal level of 'background' hCG present in urine of non-conception cycles (Wolkersdorfer *et al.*, 1998), an additional group of women was recruited, consisting of normo-ovulatory lesbian women who were receiving no hormone treatment and where not attempting to conceive. The reference group followed the same procedure for urine collection as the other participants, for one cycle only.

hCG immunoassay

The concentrations of hCG in urine were analyzed using the method described by Boomsma *et al.* (2009). In short, following sample thawing, 0.5 ml of dephosphorylated urine sample was incubated overnight in a hCG test unit (hCG kit for Immulite 1000®, Siemens, The Netherlands). After fluid aspiration from the units, the test units were analyzed in the Immulite 1000® chemistry analyser (Siemens). The functional sensitivity of the assay was 0.06 mU/ml, with 20% coefficient of variation. In order to correct for variations in urine concentration, hCG levels were corrected for creatinine concentration.

Implantation monitoring

Implantation was determined using a piece-wise linear mixed model on logarithmically transformed hCG/creatinine data. hCG values were log-transformed for analysis in the mixed linear model in order to normalize distributions and reduce the influence of outliners. The model consists of two straight lines for the per-patient trajectory of hCG over time, with the two lines crossing at the unobserved day of implantation. The indicated day of implantation was implemented into the model as a latent variable. The mixed model accounted for variation between patients in both the baseline hCG levels and the slopes of the hCG trajectories. Implantation was defined as having occurred when the slope of the estimated line after the day of implantation exceeded the maximum slope seen in the reference samples.

The following clinical end-points were defined: a clinically recognized pregnancy was detected by a positive urinary pregnancy test, performed at home when no, or aberrant, menstrual blood loss appeared. An EPL was considered to have occurred when an implantation detected by increased urinary hCG levels was followed by a negative clinical urinary pregnancy test or a normal menstrual period. An intrauterine pregnancy identified on ultrasound but lost in the first trimester was described as a miscarriage. An ongoing pregnancy was diagnosed when fetal heart activity was demonstrated on ultrasound at 9 weeks of gestation.

Statistical analysis

We investigated the rate of EPL among women with unexplained infertility versus healthy, presumed fertile women. Since the implantation rate in the unexplained infertility population was unknown, we decided to measure EPL rates per cycle rather than per implantation. Earlier studies showed an incidence of EPL of 6–10% per cycle in the first three cycles in a healthy population (Wilcox *et al.*, 1988; Wang *et al.*, 2003). In order to show a difference between groups of 20% (10 versus 30%), with 80% power and a P-value <0.05, 60 women in each group were required. Data were analyzed with SPSS 15.0 (SPSS Inc., Chicago, IL, USA). Student's t-test was used to evaluate differences in baseline characteristics. Odds ratios (ORs) and 95% confidence intervals (CIs) were calculated, for EPL using the exact method in Cytel Studio version 8.

Results

Of the 64 women recruited in the unexplained infertility study group, three dropped-out before the first cycle (Fig. 1). Thirty-eight patients collected serial urine samples for 3 cycles, 15 patients for 2 cycles and 8 for 1 cycle. Reasons for collecting urine samples for only one or two

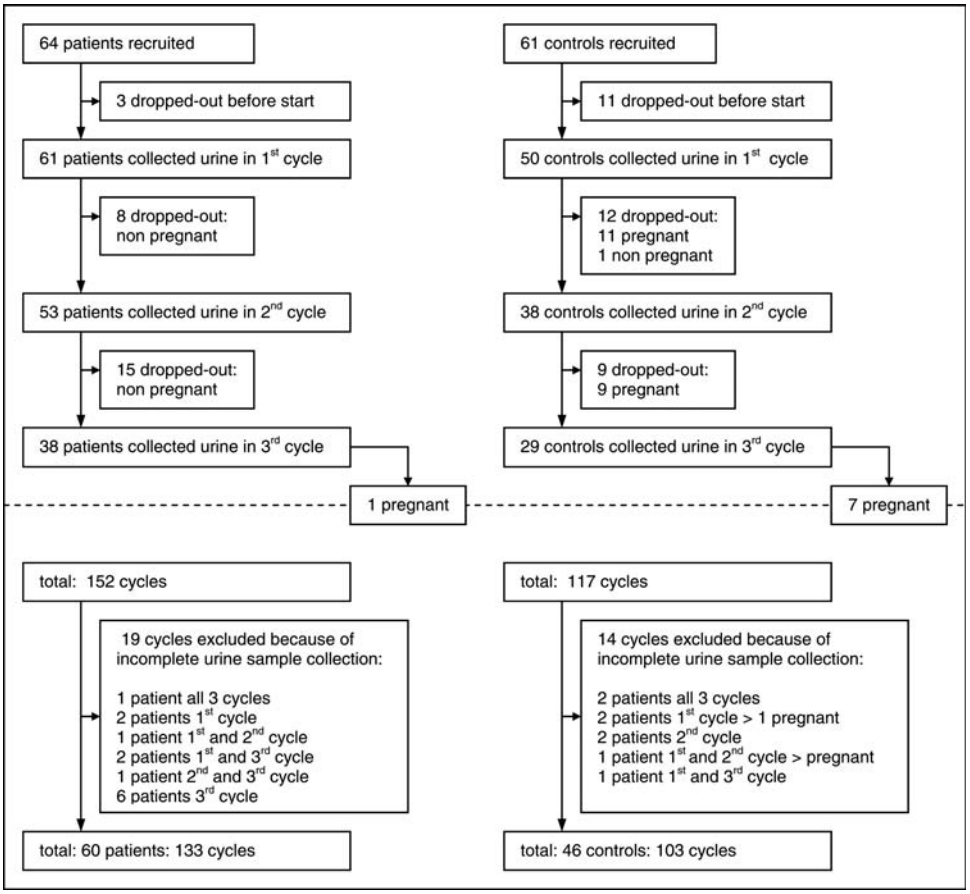

Figure 1 Number of participants enrolled in the study and included in the analysis.

cycles were either personal (holiday, work) or the opportunity to proceed quickly to IVF treatment. A total of 61 women were recruited in the control group, and 11 of them dropped-out before the first cycle. Twenty-nine participants performed 3 cycles, 9 participants 2 cycles and 12 participants 1 cycle. Reasons for collecting urine for only one or two cycles were all pregnancies, except for one patient who dropped-out for other reasons. In the patient group, 19 cycles were excluded before analysis because of incomplete urine sample collection, and in the control group, 14 cycles were excluded (including two cycles leading to pregnancy). We analyzed 133 cycles of 60 patients and 103 cycles of 46 controls. Baseline characteristics of these patients and controls are shown in Table I. The control group was significantly younger than the study population.

The reference curve of 'background hCG' was based on the log-transformed data of 12 cycles of women not attempting to conceive.

In the 133 cycles of 60 women with unexplained infertility, one implantation was detected with the linear mixed model (Fig. 2). This implantation proceeded to an ongoing pregnancy. All patients with unexplained infertility were awaiting IVF treatment. After the study period but before the start of a first IVF treatment, three more patients became pregnant. As a result of IVF treatment, 70% of remaining patients became pregnant within three attempts.

In the 103 completely collected cycles of 46 control patients, 30 implantations were detected. Analysis of urinary hCG profiles using the linear mixed model indicated that 24 clinical pregnancies and six cases of EPL occurred (Fig. 2). Of the 24 clinical pregnancies detected, 1 appeared to be a twin pregnancy and 2 ended in miscarriage. In the subsequent cycle, two of the patients who experienced EPL became clinically pregnant.

Table I Baseline characteristics of participants with unexplained infertility and healthy controls for whom hCG data were analyzed.

	Unexplained infertility ($n = 60$)	Healthy controls ($n = 46$)	*P*-value[a]
Age (years)	33.5 (±3.1)	28.7 (±3.3)	<0.0001
Cycle length (days)	28.3 (±1.8)	28.6 (±6.9)	NS
Nullipara	60%	57%	NS
Trying to conceive (years)	3.7 (±1.6)	0.1 (±0.1)	<0.0001

Values are mean ± SD.
[a]Student's *t*-test.

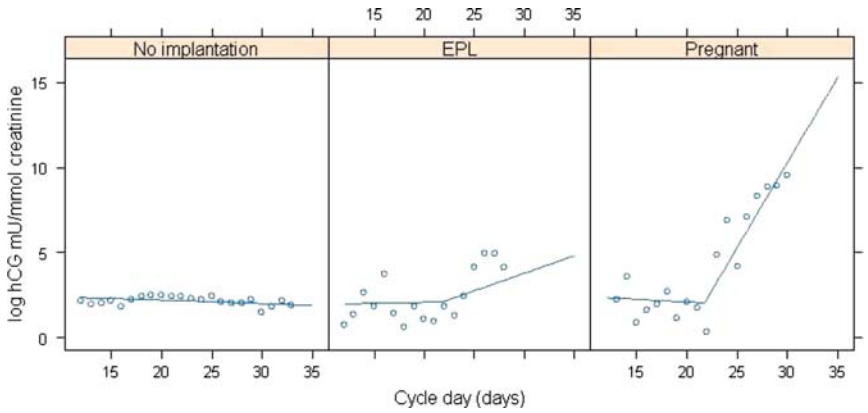

Figure 2 Example of fitted (straight line) versus observed (circles) log-transformed hCG/creatinine patterns in the situation of no implantation, EPL and ongoing pregnancy using a piece-wise linear mixed model.

The observed implantation rate in patients with unexplained infertility was 0.8% per cycle and the EPL rate in this group was 0%. The implantation rate in the control group was 29% per cycle, EPL rate was 5.8% per cycle and early clinical loss (miscarriage) was 2% per cycle. The absolute OR for achieving an implantation in the unexplained fertility versus the control group was 0.019 (95% CI: 0.003–0.145). After correction for age differences between the groups, this OR became 0.013 (95% CI: 0.001–0.106). Age therefore was not a significant confounder ($P = 0.21$). The OR of unexplained fertility versus control group for EPL per cycle was 0 (95% CI: 0–0.795, $P = 0.026$), and for EPL per implantation 0 (95% CI: 0–162).

Discussion

In this study, we aimed to test the hypothesis that recurrent preclinical pregnancy loss may present as 'unexplained' infertility; however, the results of our study of hCG levels in luteal phase urine do not support this hypothesis. In our rigorously defined group of patients with otherwise unexplained infertility, we did not observe any case of EPL. In fact, we observed only a single implantation in this group, resulting in an extremely low implantation rate of 0.8% per cycle. In the control group, consisting of healthy women who were trying to conceive, we detected implantation and EPL rates comparable with those previously reported in presumed fertile populations (Wilcox *et al.*, 1988; Wang *et al.*, 2003).

We have to conclude that the rate of EPL per cycle is not increased in patients with unexplained infertility compared with healthy controls. Moreover, implantation events were rare in the patient group. Taken together with the high rate of implantation observed when the patient group underwent IVF, it can be concluded that 'unexplained' infertility usually represents a failure of conception, preimplantation embryo development or initiation of implantation.

Previous studies of a rigorously defined group of women with unexplained infertility versus healthy controls showed subtle alterations in various hormones (i.e. FSH, LH, progesterone, estradiol and cortisol) across the menstrual cycle, suggesting a diminished ovarian reserve (Leach *et al.*, 1997). Evidence for functional abnormalities in oocyte and/or sperm function in couples with unexplained infertility come from IVF studies in which lower fertilization rates were reported compared with those with tubal factor infertility (Hull, 1994). The value of IVF treatment in the management of unexplained infertility is demonstrated by our observation that 70% of the patient group achieved a clinical pregnancy within three IVF treatments. Either by optimizing fertilization, enabling embryo selection or some other unknown mechanism, the ability to address many of the putative causes of unexplained infertility through IVF is again demonstrated (Pandian *et al.*, 2005).

The single implantation detected in the patient group resulted in an ongoing pregnancy (0.8% ongoing pregnancy rate). In Canada, in a large cohort of patients with untreated unexplained infertility, 263 conceptions resulting in a live birth occurred during 28 125 months. This shows a comparable rate of 0.9% ongoing pregnancies per cycle (Collins *et al.*, 1995).

In our study, EPL was often followed by a vital pregnancy in the subsequent cycle and is, as such, a positive predictor of subsequent fecundity. This observation is consistent with the previously published literature on this topic (Wilcox *et al.*, 1988; Wang *et al.*, 2003; Pandian *et al.*, 2005).

Although the intact hCG molecule is usually regarded as the main analyte for the detection and monitoring of early pregnancy, it can fluctuate markedly during early pregnancy (Wolkersdorfer *et al.*, 1998; McChesney *et al.*, 2005). These fluctuations, despite correction for urine concentration by creatinine levels, were visible in the majority of the cycles in our study. Whether these fluctuations were mainly caused by *in vivo* hCG production and excretion, or by the storage and detection methods, remains unclear (McChesney *et al.*, 2005).

We considered implantation to have occurred when the estimated slope of the hCG trajectory exceeded the maximum slope seen in the reference population, within the linear mixed model on log-transformed hCG/creatinine data. This definition is strict, considering that there were significant variations in hCG levels in the reference group. By using this strict definition, we may have underestimated the number of implantations that were initiated. However, this model was shown to be 100% sensitive and specific for those cycles with known conceptive status.

Respectively, 12.5 and 8.5% of the cycles in the study and control group were excluded because of incomplete urine sample collection.

Cycles were excluded when samples from the period covering the possible 'window of implantation' were missing. Statistical methods to estimate curves for incomplete cycles were described previously (Wang *et al.*, 2003); however, a ±10% loss of cycles was considered acceptable for the purposes of this study.

In the interpretation of these findings, a number of caveats need to be considered. The first derives from the significant difference in age between women participating in the healthy control group and the women in the study population (mean 28.7 versus 33.5 years). This reflects the difficulty in finding sufficient identically age-matched volunteers for this study. However, after correction for age, significant differences in implantation and EPL rates were still evident. The percentage of nulliparous women was equal in both groups, suggesting that most women started trying to conceive around the same age; however, after a mean of 3.7 years, women in the patient group were still trying.

Secondly, the women participating in the healthy control group were presumed to be fertile. While some had conceived previously, the majority was nulliparous; so fertility was not proven. However, they all had a regular cycle and with no known fertility problems; they were more representative of the general population than a proved fertile subgroup.

Thirdly, as ovulation detection was not performed in this study, the frequency of anovulatory cycles in each group is not known. However, all participants reported regular cycles and patients with unexplained infertility had demonstrated a normal midluteal progesterone level as part of their infertility investigations.

Finally, most patients had suffered from infertility for several years and all had undergone at least three unsuccessful IUI treatments before presenting. IUI in natural cycles or in combination with mild ovarian stimulation is considered an effective treatment for unexplained infertility, with pregnancy rates per couple of 18–33% (Guzick *et al.*, 1999). The patient group included in this study had all failed to conceive with IUI treatment and therefore represents a cohort of clearly subfertile couples referred for tertiary care, and caution is needed before generalizing our results to patients in whom unexplained infertility has recently been diagnosed. On the other hand, the clear phenotype defined as unexplained infertility represents a strength of this study as does the large and complete set of data generated from daily urine samples obtained in 236 cycles.

In summary, among the population of patients with unexplained infertility studied here, no post-implantation EPLs were observed and implantation was detected only once. In the control group, implantation and EPL rates were consistent with those reported in previous studies. These data allow us to conclude that post-implantation failure is unlikely to be an important 'cause' of unexplained infertility.

Authors' roles

Y.E.M.K.: acquisition, analysis and interpretation of data; manuscript drafting and critical discussion. C.M.B.: study design; patient recruitment and acquisition of data; critical discussion. M.J.C.E.: analysis and interpretation of data; critical discussion. E.G.W.L: hCG immuno analysis; critical discussion. N.S.M: study design; analysis and interpretation of data; manuscript drafting and critical discussion.

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References

Barlow P, Lejeune B, Puissant F, Englert Y, Van RM, Degueldre M, Vekemans M, Leroy F. Early pregnancy loss and obstetrical risk after *in-vitro* fertilization and embryo replacement. *Hum Reprod* 1988; **3**:671–675.

Boomsma CM, Kavelaars A, Eijkemans MJ, Amarouchi K, Teklenburg G, Gutknecht D, Fauser BJ, Heijnen CJ, Macklon NS. Cytokine profiling in endometrial secretions: a non-invasive window on endometrial receptivity. *Reprod Biomed Online* 2009;**18**:85–94.

Canfield RE, O'Connor JF, Birken S, Krichevsky A, Wilcox AJ. Development of an assay for a biomarker of pregnancy and early fetal loss. *Environ Health Perspect* 1987;**74**:57–66.

Chard T. Frequency of implantation and early pregnancy loss in natural cycles. *Baillieres Clin Obstet Gynaecol* 1991;**5**:179–189.

Collins JA, Burrows EA, Wilan AR. The prognosis for live birth among untreated infertile couples. *Fertil Steril* 1995;**64**:22–28.

Delbaere I. Implantation of the human embryo: clinical and epidemiological aspects. *Verh K Acad Geneeskd Belg* 2008;**70**:257–283.

Guzick DS, Carson SA, Coutifaris C, Overstreet JW, Factor-Litvak P, Steinkampf MP, Hill JA, Mastroianni L, Buster JE, Nakajima ST *et al.* Efficacy of superovulation and intrauterine insemination in the treatment of infertility. National Cooperative Reproductive Medicine Network. *N Engl J Med* 1999;**340**:177–183.

Hakim RB, Gray RH, Zacur H. Infertility and early pregnancy loss. *Am J Obstet Gynecol* 1995;**172**:1510–1517.

Hull MG. Effectiveness of infertility treatments: choice and comparative analysis. *Int J Gynaecol Obstet* 1994;**47**:99–108.

Leach RE, Moghissi KS, Randolph JF, Reame NE, Blacker CM, Ginsburg KA, Diamond MP. Intensive hormone monitoring in women with unexplained infertility: evidence for subtle abnormalities suggestive of diminished ovarian reserve. *Fertil Steril* 1997;**68**:413–420.

Macklon NS, Geraedts JP, Fauser BC. Conception to ongoing pregnancy: the 'black box' of early pregnancy loss. *Hum Reprod Update* 2002; **8**:333–343.

McChesney R, Wilcox AJ, O'Connor JF, Weinberg CR, Baird DD, Schlatterer JP, McConnaughey DR, Birken S, Canfield RE. Intact HCG, free HCG beta subunit and HCG beta core fragment: longitudinal patterns in urine during early pregnancy. *Hum Reprod* 2005; **20**:928–935.

Moghissi KS,, Wallach EE. Unexplained infertility. *Fertil Steril* 1983; **39**:5–21.

Pandian Z, Bhattacharya S, Vale L, Templeton A. *In vitro* fertilisation for unexplained subfertility. *Cochrane Database Syst Rev* 2005;CD003357.

Sharp NC, Anthony F, Miller JF, Masson GM. Early conceptual loss in subfertile patients. *Br J Obstet Gynaecol* 1986;**93**:1072–1077.

Smith S, Pfeifer SM, Collins JA. Diagnosis and management of female infertility. *JAMA* 2003;**290**:1767–1770.

Templeton AA,, Penney GC. The incidence, characteristics, and prognosis of patients whose infertility is unexplained. *Fertil Steril* 1982;**37**:175–182.

Wang X, Chen C, Wang L, Chen D, Guang W, French J. Conception, early pregnancy loss, and time to clinical pregnancy: a population-based prospective study. *Fertil Steril* 2003;**79**:577–584.

Wilcox AJ, Weinberg CR, Wehmann RE, Armstrong EG, Canfield RE, Nisula BC. Measuring early pregnancy loss: laboratory and field methods. *Fertil Steril* 1985;**44**:366–374.

Wilcox AJ, Weinberg CR, O'Connor JF, Baird DD, Schlatterer JP, Canfield RE, Armstrong EG, Nisula BC. Incidence of early loss of pregnancy. *N Engl J Med* 1988;**319**:189–194.

Wolkersdorfer GW, Bornstein SR, Hilbers U, Zimmermann G, Biesold C, Lehmann M, Alexander H. The presence of chorionic gonadotrophin beta subunit in normal cyclic human endometrium. *Mol Hum Reprod* 1998;**4**:179–184.

World Health Organization. *WHO Laboratory Manual for the Examination of Human Semen and Sperm-Cervical Mucus Interaction*, 4th edn. Cambridge: Cambridge University Press, 1999, 128.

Zinaman MJ, Clegg ED, Brown CC, O'Connor J, Selevan SG. Estimates of human fertility and pregnancy loss. *Fertil Steril* 1996;**65**:503–509.