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Neuroendocrine axis and reproduction

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Neuroendocrine axis and reproduction

Introduction

The physiology of the female reproductive system is regulated by a complex neuro-hormonal interaction whose operative center is the hypothalamus-pituitary-ovarian axis.

The evidence that neurons poured into the general circulation biologically active substances (neurotransmitters and neuromodulators) represents the physio-anatomic substrate of the neuroendocrine complex as an integrated functional unit. The presence of structures in the nerve receptors for different hormones, capable, in their turn, to modulate the neuronal activity, reinforces this assumption.

This delicate interplay is affected by external and internal stimuli that can alter its correct operation, deviating from physiology to pathology.

This PhD thesis is based on the conduct of two lines of research focused on alterations in the hypothalamic-pituitary-gonad axis functioning. The two most common disorders of ovarian function, micropolycystic ovary syndrome (PCOS) and endometriosis, have been identified as models to assess the impact, on the axis, of endogenous and environmental variables, respectively.

The first line of research has been focused on the hypothesis that the two main forms of clinical expression of PCOS, the strictly linked to hyperandrogenism and that one associated to insulin resistance, represent distinct disease entities and are, consequently, supported by different pathogenetic mechanisms. This hypothesis finds its origin on the fact that these two forms determine distinguishable endocrine and metabolic profiles. Nevertheless, there are no studies
investigating whether the PCOS phenotype differently alters the morphology gonads. On this basis, a clinical observational study has been conducted in PCOS women, aimed at evaluating the relationship between ultrasound pictures of polycystic ovaries and the endocrine-metabolic state of patients.

The second line of research stems from the hypothesis of a possible role of environmental pollutants on another of the most common reproductive age pathologies: endometriosis. The disease affects 6-10% of women and its exact etiology remains unknown. The possible role of toxic agents was proposed back in 1993 by Rier and collaborators and, since then, the interest in the impact on it of contaminants has been high. A systematic review of literature through electronic database was conducted without time restriction in order to analyze the relation between endometriosis and the most common environmental pollutants.

The PhD thesis is organized in three chapters. The first two are aimed to report results from the main lines of research. A third chapter regards a parallel research experience that the PhD student started in the last year, during her fellowship at the University “Mangiagalli” of Milan (Italy). Each chapter is further divided in a first part concerning what’s is known for any single topic and a second one dedicated to related studies done during the PhD.
Chapter I: The role of metabolic (endogenous) variables on the function of the gonadal axis: The PCOS model

1. The Polycystic Ovary Syndrome

1.a Epidemiology, risk factors and main endocrine alterations

Polycystic ovary syndrome (PCOS) is a common endocrine disease affecting approximately 5-10% of women of reproductive age (Consensus, Fertil Steril, 2008). This syndrome is considered a multifactorial disease and the individual susceptibility is probably determined by multiple genetic and environmental risk factors. The etiology of the syndrome remains obscure, and the variability in phenotype expression continues to render the clinical care and research concerning this heterogeneous condition challenging.

Whatever the cause, the syndrome appears to be characterized by a wide variety of hormonal changes.

We will analyze them individually.

FSH deficiency

In patients with PCOS we observe lower levels of serum FSH compared to women with normal ovulation. Although strongly reduced serum levels of FSH rarely occurs, it has been demonstrated
that, during PCOS, they are below the threshold required to stimulate maturation of the follicle. FSH suppression leads to a greater accumulation of antral follicles with diameter between 2 and 8 mm. The mechanisms underlying the pathogenesis of this anomaly remain unknown. Nevertheless, there is an increasing body of evidence supporting the hypothesis of inherent differences in the mechanisms of folliculogenesis. Due to constantly reduced levels of FSH, folliculogenesis is obstructed and the growing follicles hesitate in liquid cysts. The premature end of growing follicles interferes with the dominant follicle selection process causing anovulation.

**Hypersecretion of LH**

Only in the 10-20% of women with a history of PCOS, the values of LH and FSH are normal. In the remaining portion, a significant increase in LH levels is observed since the early follicular phase. As a consequence, a reversal of the LH/FSH ratio occurs, which reaches values approximated at about 2.5. This phenomenon has to be found in different causes. First, regardless of the primary cause, in PCOS is observed hyperandrogenism; the increase in circulating androgens establishes a persistent positive feedback at pituitary level and, therefore, a maintenance of the hypersecretion of LH. Both factors are mutually reinforcing. In presence of a high concentrations of androgens, LH-related suppression of estrogens and progesterone regulation of hypothalamic release of GnRH seem to be inhibited. Another possible cause is an increase in the pulses of GnRH at the hypothalamic level. This phenomenon is probably promoted by an interplay between androgens and some endogenous modulators and results in a significant increase in the amplitude and the frequency of the pulses of LH.

Insulin also represents a chronic stimulus to LH production. In fact, it has a steroidogenic activity and it is able to synergize the action of LH on thecal cells, resulting in increase in the synthesis of androgens.

The increase in LH levels during the folliculogenesis results in a suppression of FSH and consequent alteration of the function of the granulosa cells. In physiological conditions, the granulosa cells are sensitive to LH only when the follicle has reached a diameter of about 9-10 mm. In contrast, in women with PCOS, this response is anticipated, involving also small follicles of about 4 mm. Therefore an early luteinization and the atresia of small follicles occur.

The final effect is the premature maturation of oocytes with chronic anovulation.

**Hyperandrogenism**

Hyperandrogenism is the most common endocrine abnormality in PCOS, involving 60-80% of women affected. The major circulating androgens are essentially produced by two separate sources:
the ovary and the adrenal gland. The ovary is the main source of androstenedione and testosterone, and the adrenal gland produces mainly Dehydroepiandrosterone sulfate (DHEA-S) as well as testosterone.

The multiple effects of androgen hormones are due to the free part, namely that is not linked to Sex Hormone Binding Globulin (SHBG), a plasma protein produced in the liver. The circulating androgens derive either directly from an increase of the ovarian biosynthesis either by a reduction of SHBG, as it often happens in patients with insulin-resistance. The same androgens reduce SHBG with direct mechanism. The increased concentration of androgens in the follicular fluid is associated with increased serum levels of LH, which may help to stop the development of the dominant follicle leading to block and degeneration. It is also suggested that high levels of androgens have a negative effect on the development of oocyte competence. This phenomenon is determinant in the establishment of a condition of chronic anovulation.

The high concentrations of testosterone at the peripheral level make it available for its transformation to dihydrotestosterone, which is the biologically most effective hormone. This conversion is performed by the 5-alpha reductase, which is especially expressed at the cutaneous level. The result is an increased hair growth at the level of normally hairless regions like face, breast areola, linea alba and back.

1.b Diagnostic criteria

Proposed diagnosed criteria for PCOS include the NIH Consensus, in 1990, that defined it as the presence of clinical and/or biochemical hyperandrogenism and oligomenorrhea/anovulation. Later, in 2003, the Rotterdam Consensus (Fauser et al., 2012) introduced the polycystic ovary appearance (PCO) on ultrasound as a new criterion to be added to the two previous criteria of the NIH, and the diagnosis requires two out of these three criteria. In turn, the Androgen Excess and PCOS Society considered that androgen excess is a central event in the pathogenesis and development of PCOS, and established that this criterion should be present and accompanied by one of the others: oligomenorrhea and/or PCO. In all cases, exclusion of other androgen excess disorders, such as non-classical congenital adrenal hyperplasia (NC-CAH), Cushing’s syndrome, androgen-secreting tumors, hyperprolactinemia, thyroid diseases, drug-induced androgen excess, should be excluded, as well as other causes of oligomenorrhea or anovulation.
In consequence, new phenotypes have arisen in addition to the classic phenotype, in which patients present hyperandrogenism and oligomenorrhea with or without PCO on ultrasound. These new phenotypes are the “ovulatory phenotype”, which means hyperandrogenism and PCO in an ovulatory woman, and the “non-hyperandrogenic phenotype”, in which there is oligomenorrhea and PCO, without overt hyperandrogenism. Anti-Müllerian Hormone (AMH) levels are correlated with follicle counts and its measurement has been useful for screening and prognosis of reproductive issues. The determination of an AMH cutoff value is still lacking, but may become an additional tool to define PCO and PCOS phenotypes in the near future. In turn, morphological ovarian changes are not exclusive of PCOS, and the presence of PCO in no hirsute women with normal cycles is not negligible, varying from 2.5 to 33% in different studies. In addition, while the inclusion of a non-hyperandrogenic phenotype of the diagnosis of PCOS is still controversial, some authors consider the presence of PCO as being itself a sign of hyperandrogenism. Recently, an Expert Panel from a NIH Evidence-Based Methodology Workshop on PCOS reinforced the use of the wider Rotterdam Criteria to diagnose the Syndrome. Classic PCOS is the most common phenotype, with a prevalence of around 70%, with the ovulatory and the non-androgenic phenotypes sharing the other 30% of prevalence. Clinical characterization also changes throughout the lifespan, especially during the post-menarche years and in the menopause transition.
Analyzing these criteria it’s clear that the scientific community didn’t take into consideration the main endocrine alterations typical of PCOS. In particular, the alterations that involve the neuroendocrine axis, such as LH hypersecretion, and the metabolic features do not appear either in the form of minor criteria.

So it should be stressed that the choice of the individual laboratory tests and instrumental investigations should be personalized and remains subject to a proper medical history framework and a complete physical examination.

**Chronic anovulation**

The first aspect that should be considered in the diagnostic evaluation of a patient with suspected PCOS is anovulation.

To validate the presence of ovulation we use ultrasound and hormonal investigations. Through serial sonograms, repeated at regular intervals, you can evaluate follicle growth and changes that occur in endometrium. The hormonal dosages, on the other hand, are performed to check that corresponds to the adequate follicular growth hormone production. The dosage of progesterone, to be carried out around the 20-25th day of the cycle, according to ultrasonographic evidence, appears to be diriment for the diagnosis of occurrence of ovulation: levels greater than 5 ng/ml confirm this phenomenon. Before the 14th day can be indicated the detection of serum E2 and LH in order to assess the presence of the pre-ovulatory peak.
Hyperandrogenism

The second aspect is clinical and biochemical hyperandrogenism.

It’s important to measure hormones in early follicular phase. Elevated free Testosterone (T) levels are observed in approximately 70% of PCOS patients. The vast majority of the abnormal values are in the form of free T, with the sole measurement of total T adding little to the diagnosis. The present recommendation is to measure free T concentration either directly by equilibrium dialysis, or to calculate free T based on the total T measured accurately (by RIA using column chromatography, or by LC-MS or GC-MS) and SHBG (measured using competitive binding or a high quality immune-based assay). The value of measuring Androstenedione is unclear, but it may increase the number of subjects identified as hyperandrogenic by 10%. Approximately 20% to 30% of patients with PCOS will demonstrate abnormal levels of DHEAS, which may be the only abnormality in circulating androgens in 10% of these patients. Nonetheless, we should note that DHEAS levels might not always reflect the status of adrenocortical steroidogenesis, and over interpretation of DHEAS levels should be avoided. Circulating levels of DHEA have limited diagnostic value. Serum measurements of androgens, including free T, should be used only as an adjuvant tool for the diagnosis of hyperandrogenic disorders, and never as the sole criterion for diagnosis or in lieu of the clinical assessment. This latter recommendation reflects the fact that between 20% and 40% of women with PCOS will have androgen levels within the “normal” range, and assays for androgens, particularly total T, are highly variable and inaccurate.

Clinical features of hyperandrogenism frequently seen in PCOS include hirsutism, acne, and androgenic alopecia.

Hirsutism is the presence of terminal hairs on the face and/or body in a female in a male-type pattern. The most common method of determining the presence of hirsutism uses a visual score. Various methods have been proposed. The most commonly used method is a modification of a method originally reported by Ferriman and Gallwey. Nine body areas, including the upper lip, chin, chest, upper back, lower back, upper and lower abdomen, upper arm, and thigh, are assigned a score of 0–4 based on the density of terminal hairs. A score of 0 represented the absence of terminal hair, a score of 1 minimally evident terminal hair growth, and a score of 4 extensive terminal hair growth. The cutoff value should be established after the study of a large population of unselected women. Using this approach, cutoff values for defining hirsutism have been variously reported to be a score of 6 or greater, 7 or more, and 8 or more. Overall, hirsutism is an important feature of PCOS, affecting approximately 65% to 75% of patients with PCOS, including women of White,
Black, and Southeast Asian race. The prevalence of hirsutism in PCOS is likely to be less among women of East Asian extraction.

Fig.1 Ferriman and Gallwey score

Acne affects approximately 12% to 14% of White PCOS patients although the prevalence of this dermatologic abnormality varies with ethnicity: it is reportedly higher in Asian Indians and lower in Pacific Islanders it is unclear whether the prevalence of acne is significantly increased in these patients over that observed in the general population.

Scalp hair loss in women is a distressing complaint with significant psychologic morbidity. It usually represents the pilosebaceous unit response to endogenous androgens and may be associated with acne and hirsutism. Androgen sensitivity of the pilosebaceous unit varies, and there is poor correlation between clinical features and evidence of biochemical hyperandrogenism. The presence of DHT, formed from the 5a-reduction of the dermal papilla, is associated with a higher 5a-reductase activity in the hairs plucked from a scalp presenting with androgenic alopecia. In addition to androgen excess, other potential etiologies of alopecia or diffuse scalp hair loss in any woman may be genetic (familial premature scalp follicular loss), environmental (damage following the use or abuse of hair cosmetics), and nutritional (e.g., poor protein intake, zinc deficiency, iron-deficient anemia). Androgenic alopecia is a recognized sign of PCOS. However, the prevalence of this abnormality in PCOS is unclear. The pattern of hair loss in PCOS generally involves thinning of the crown with preservation of the anterior hairline. Androgenic related alopecia in women with PCOS tends to be seen in the anterior midvertex area extending to the crown. The anterior hairline remains intact in women with PCOS and significant a bitemporal scalp hair recession is unusual except in virilizing syndromes. The only presence of alopecia or diffuse scalp hair loss in women may be the only dermatologic sign of PCOS. However, estimates regarding the prevalence of alopecia in PCOS vary widely, from 5% to 50%, and further studies are needed to better define this prevalence.
In addition to PCOS, there are numerous other disorders of androgen excess in women, including the adrenal hyperplasia (CAHs), syndromes of severe insulin resistance, and androgen- secreting neoplasms (ASNs); and disorders that have not been well identified (e.g., idiopathic hyperandrogenism) or that have the appearance of androgen excess (e.g., idiopathic hirsutism). These disorders account for approximately 10% to 30% of all patients with androgen excess. There are also a number of other disorders that may result in ovulatory dysfunction, including hyperprolactinemia and thyroid abnormalities. Consequently, although PCOS has specific diagnostic criteria, other disorders associated with androgen excess and/or menstrual irregularities should be excluded. First of all it is important to assess serum levels of testosterone. In the presence of concentrations greater than 2 ng/ml it is possible to suspect a disease of neoplastic origin, especially when the condition of hyperandrogenism is established in a short time (2-6 months). To rule out congenital adrenogenitale syndrome, linked to enzyme deficiency primarily of 21-hydroxylase, it must be taken into account that it manifests in prepuberal age and usually is associated with signs of virilization, while in adrenogenitale acquired immune deficiency syndrome, due to adrenal-secreting tumors, the picture clinical manifests itself in extremely short times. In differential diagnosis, it is important the dosage of 17-OH progesterone. Normally the 17-OH progesterone of ovarian origin tends to increase during the second phase of the menstrual cycle as it is synthesized by the corpus luteum. Although the hormone frequently appears higher in patients with PCOS, the detection of values above 200 ng/dL, in the follicular phase, justifies diagnostic exams necessary to rule out alternative endocrine dysfunction such as adrenal hyperplasia. In these cases, it is indicated to perform a stimulation test with ACTH.

**Ultrasound pattern**

The third criterion considered in the Consensus Conference of Rotterdam is the ultrasound pattern of the ovary.

According to the actual recommendations, the criteria that have high sensibility and specificity to define the presence of PCOS are:

- an ovarian volume >10 cm$^3$;
- 12 or more follicles;
- Follicles diameter of 2-9 mm.
Taking into account only the indications of Rotterdam Consensus Conference, it’s evident that the ultrasound diagnosis considers only the valuation of ovarian volume and the number of follicles. Individual variables such as the follicles distributions, the stroma expansion and vascular characteristics of the organ are excluded.

The classic aspect of a polycystic ovary is represented by a predominantly elongated body shape and with plenty of stroma in the intrathecal region; the follicles visible on ultrasound as a hypo-anechoic areas with a round shape, are found mainly on the outskirts of the organ and are arranged according to an architecture defined by some authors as "necklace" or "string of pearls". Even the presence of a single ovary with these features allows the diagnosis of PCOS.

In the attempt to increase the diagnostic efficacy of ultrasound, Fulghesu et al. (2001) have proposed the enlarged ovarian stroma as a marker of PCOS (Pache et al., 1992; Dewailly et al., 1994). The stroma/total area (S/A) has a sensitivity of 100% and is highly correlated with plasma androgen level. It’s considered positive, for the morphological diagnosis, a cut-off of 0.34.

The coexistence of different criteria and the continuous research for ultrasound parameters reflect the climate of uncertainty and the need to identify new ways to increase the sensitivity of ultrasound diagnosis.

1.c Metabolic alterations

Obesity is a prevalent characteristic of PCOS, ranging from 12.5% to 100%, with a pooled estimated prevalence of 49%, as shown by a recent meta-analysis. The presence of obesity may exacerbate the metabolic and reproductive disorders associated with the syndrome, including insulin resistance, dyslipidemia and metabolic syndrome. A meta-analysis has shown that women with PCOS have higher levels of triglycerides (TG), LDLcholesterol and total cholesterol (TC), and lower HDLcholesterol levels compared with control women, regardless of body mass index (BMI). In addition, PCOS women present higher risk for type 2 diabetes. PCOS is also associated with a clustering of cardiovascular risk factors. However, there is no definitive evidence for increased cardiovascular events, or data showing that PCOS alone leads to increased cardiovascular risk independent of associated risk factors. In fact, more rigorous cohort studies of long-term cardiovascular outcomes and clinical trials of risk factor modification are required for women with PCOS.
The Ultrasonographic findings related to insulin resistance in patients with polycystic ovarian syndrome: a retrospective observational study

2.1 Scientific BACKGROUND

Although insulin resistance is found in a high percentage of women affected by PCOS (Diamanti-Kandarakis et al., 2012), neither hyperinsulinism nor the metabolic syndrome are among the diagnostic criteria for PCOS established by the Rotterdam Consensus Group (Revised 2003 consensus, *Fertil Steril*, 2004). Furthermore, the diagnostic criteria for insulin resistance are much debated.

There is evidence that the pathogenic mechanisms of insulin resistance-related PCOS differs from those underlying hyperandrogenism (Diamanti-Kandarakis et al., 2012; Diamanti-Kandarakis et al., 2006). This assumption has allowed to identify two distinct PCOS phenotypes: one more strictly due to hyperandrogenism and a second where insulin resistance plays a crucial pathogenetic role. Currently, the "gold standard" procedure for the diagnosis of insulin resistance is the euglycemic clamp together with the so-called "minimal model". A less time-consuming method is homeostasis model assessment (HOMA). It has been demonstrated that the co-presence of increased HOMA and alterations of anthropometric parameters (body mass index [BMI] and the waist-to-hip ratio [WHR]) has high accuracy in predicting abnormal “minimal model” and can be adopted, in the clinical practice, as reliable method of diagnosis (Ciampelli et al., 2005).

According to Rotterdam Criteria, polycystic appearance of the ovary is diagnosed when more than 12 follicles and/or a volume >10 ml is detected in at least one gonad. Nevertheless, no clear identification of different morphologic patterns is considered. It could be argued that, if hyperandrogenism and hyperinsulinism affect folliculogenesis throughout different pathogenic pathways, a difference in the size and distribution of atretic follicles may occur. This issue has never addressed before, where no distinction between ultrasound ovarian pattern in this two different PCOS phenotypes has been reported. The aim of the retrospective observational study was to assess the impact of insulin resistance on ovarian ultrasonographic parameters in patients with PCOS.
2.b Patients and Methods

Patients

We evaluated PCOS patients who fulfilled Rotterdam criteria (Revised 2003 Consensus, Fertil Steril, 2004, attending the clinics of Endocrinology of Reproduction and Sterility-Infertility of our Department from January 2013 to December 2015. Given the observational and retrospective character of this study, Ethics Committee approval was not required. Inclusion criteria were signs of polycystic ovaries, according to the most recent ESHRE/ASRM consensus criteria (Fauser et al., 2012; Balen et al., 2003). Exclusion criteria were: basal follicle stimulating hormone (FSH) >10 IU/l; administration of estrogen-progestin or other hormonal treatment in the previous six months; congenital adrenal hyperplasia and other endocrine abnormalities; presence of ovarian formations with diameters >14 mm in two ultrasound examinations carried at an interval of 30 days; thyroid disorders; diabetes mellitus; presence of a single ovary; and previous ovary surgery.

The patients anthropometric characteristics (weight, height, BMI and WHR) were collected as well as serum levels of gonadotrophins, DHEA-S and free testosterone measured in the early follicular phase. We also evaluated hirsutism with the Ferriman-Gallwey clinical score. All enrolled subjects underwent a 75-g oral glucose challenge and insulin measurements at baseline and 60, 120 and 180 minutes thereafter. Insulin resistance was diagnosed only when all the following criteria were fulfilled: HOMA >2.5 (Ciampelli et al., 2005); BMI >27 kg/m²; WHR >0.85 (Qiao et al., 2010); sex hormone binding globulin (SHBG) serum levels below the 25th percentile (Ciampelli et al., 2005; Qiao et al., 2010) and serum insulin >150 IU/ml at 60 min and a change <20% between 60 and 120 min after oral glucose load with 75 g of glucose (Matsuda et al., 1999). Based on these parameters, we identified a group of patients with insulin resistance (group 1) and a group without insulin resistance (group 2). We did not evaluate patients with intermediate metabolic conditions, namely those who did not fulfill all the criteria.

Ultrasound parameters

Pelvic ultrasound examination was carried out with a 6.5 MHz vaginal probe. The external circumference of the ovary and the stroma were measured to determine the S/A ratio (Figure 2). The following sonographic parameters were recorded for each patient: total number of ovarian follicles with a diameter <10 mm; number of follicles with diameter between 2 and 4 mm; the S/A ratio calculated with an ovarian median scan (Fulghesu et al., 2001); and ovarian longitudinal (A),
transverse (B) and coronal (C) diameters and volume (1/2 x [A x B x C]) (Fulghesu et al., 2007). Based on these parameters, we arbitrarily defined two ultrasound patterns (Figures 3 and 4): type A, characterized by >50% follicles with diameters between 5 and 9 mm and with an S/A >0.34 (“necklace” sign defined by a hyperechoic central area and a rosary-like peripheral disposition of follicles); and type B, characterized by >50% follicles measuring 2-4 mm and with an S/A ≤0.34 (no "necklace" sign and ubiquitously distributed follicles). Intermediate ultrasound patterns were not included in our analysis.

Statistical analysis

Results are reported as means ± standard deviation (SD). The student T test or the Mann-Whitney U test were used for continuous variables with a parametric or not parametric distribution, respectively. Normal distribution of continuous variables was evaluated with the Shapiro test. Cohen’s kappa was used to evaluate the agreement between sonographers with respect to ultrasound ovarian pattern. Sensitivity, specificity, positive predictive value, negative predictive value, positive and negative likelihood ratio were calculated with the MedCalc statistic software to assess the accuracy of an ultrasound pattern in identifying PCOS patients with insulin resistance. The \( \chi^2 \) test was used to compare categorical data. A \( p <0.05 \) was considered statistically significant. The SPSS, statistical software 18.0 (SPSS Inc., USA) was used to analyze data.
2.c Results

Of the 309 patients affected by PCOS admitted to our institute between December 2010 and December 2013, only women without an intermediate ultrasound profile and fulfilling the Rotterdam criteria for PCOS were included for a total of 78 patients enrolled (Figure 2). Forty-one of these women met all the criteria of insulin resistance (group 1); the remaining 32 patients did not fulfill any insulin resistance criterion (group 2). Woman with an intermediate metabolic profile were not included in our analysis (Figure 2). Demographic, anthropometric, hormonal and ultrasound features of the two groups stratified according to insulin resistance are listed in Table 1. There was no difference in terms of age, androgen levels or Ferriman-Gallwey score between the two groups. Women affected by insulin resistance were more likely to have a type B ultrasound pattern than women without insulin resistance (36/41 87.8% versus 21.8%, \( p < 0.01 \)).

The accuracy of the type B ultrasound pattern to identify PCOS women with insulin resistance was as follows: sensitivity 0.88 CI 95% (0.74–0.96); specificity 0.78 CI 95% (0.60–0.91), positive likelihood ratio 4.01 CI 95% (2.06–7.80), negative likelihood ratio 0.16 CI 95% (0.07–0.36); positive predictive value 0.84 CI 95% (0.69–0.93), negative predictive value 0.83 CI 95% (0.65–0.94). A Cohen’s kappa of 0.81, estimated in 50 subjects, indicated an excellent concordance between sonographers.

A type A ovarian pattern was found in 33 women and the type B ovarian pattern in 45 women. The demographic, anthropometric and hormonal data of patients divided according the ultrasound pattern of PCOS are listed in Table 3. The mean age of patients, serum levels of gonadotropin, androgens and the Ferriman-Gallwey score did not differ significantly between the two groups. Conversely, BMI, WHR and HOMA values were significantly higher in patients with a B type ultrasound pattern. Patients with an A type pattern had higher SHBG levels (27.2 ± 11.3 nmol/l versus 52.2 ± 21.1 nmol/l, \( p < 0.01 \)) and a larger ovarian volume (14.6 ± 5.6 versus 11.9 ± 4.1 cm\(^3\), \( p < 0.05 \)) than did patients with an A type pattern.
2.d Discussion

This line of research has shown, for the first time, a variability in the morphological alterations of the ovaries in women with PCOS and that this heterogeneity is related to different expression of hyperandrogenic and hyperinsulinaemic components. This evidence strongly supports the hypothesis that, despite converging in apparently similar clinical features, diverse endogenous variables may differently alter the neuroendocrine-ovarian axis, reinforcing the idea that hyperandrogenic and metabolic phenotypes of the PCOS may be related to different pathogenic pathways.

Body mass index, WHR and HOMA were significantly lower in patients with a type A ovary, namely those with the classical “necklace” sign, than in women with a type B ovary. A high HOMA related to elevated anthropometric indices was previously reported to be an efficient positive predictor of insulin resistance (Ciampelli et al., 2005). The association between insulin resistance and specific ultrasound patterns is supported by data obtained stratifying our study population into patients with insulin resistance (group 1) and patients without insulin resistance (group 2). Type B ovaries were significantly more frequent in group 1 than in group 2. On the contrary, the classic ultrasound picture of PCOS (type A) was more frequent in group 2 patients. Neither serum concentrations of androgens nor the Ferriman-Gallwey score differed between the two groups. However, all biochemical and clinical androgenic variables, including basal luteinizing hormone (LH), were higher in patients with a type A ovary than in those with a type B ovary, and the difference was almost significant in the case of 17-OH P concentrations (Table 3).

The different ovarian profiles observed in this study support the concept that the pathogenesis and the clinical phenotype could differ between PCOS patients with hyperandrogenism and normal anthropometric parameters and PCOS patients with insulin resistance. In other words, two physiopathogenetic pathways, one characterized by hyperandrogenism and the other by insulin resistance, could induce the same effects. They could interfere with selection mechanisms of the dominant follicle and also induce atresia of secondary follicles. These folliculogenesis changes could result in anovulation, arrest of multiple follicles at different developmental stages and hyperandrogenism, which could be either primitive or secondary depending on clinical conditions.

Based on the preliminary data, the classical ultrasound image characterized by a hyperechoic central area and a peripheral neck-lace arrangement of follicles is more typically observed in PCOS patients who have a more pronounced hyper-androgenic profile, minimal or absent insulin resistance and pronounced hypertricosis.
Ovarian morphology might change dramatically should the *primum movens* of PCOS development be insulin resistance. Normally, insulin, by way of the classic mechanism of "spill-over", binds IGF-1 receptor thereby exerting mitogenic effects on the granulosa and theca. IGF-1 plays a pivotal role in the FSH-mediated proliferation of the granulosa, and it is hence important for the growth and development of follicles (Lucy *et al*., 2011). If the proliferation of antral follicles is not finely regulated, the mechanisms governing the selection of the dominant follicle could be deranged thereby fostering follicle atresia. Our data suggest that the growth of antral follicles is blocked before in PCOS women with insulin resistance. In fact, type B ovaries are characterized by a predominance of follicles with a diameter measuring between 2 and 4 mm. In addition, lack of prominent hyperandrogenism could explain absence of hyperthecosis, which in turn may lead to a ubiquitous distribution of follicles. Although insulin production could reduce SHBG concentrations and so it could lead to an increase in the free forms of various androgens particularly testosterone (Fauser *et al*., 2012), this effect does not seem sufficient to induce the hyperthecosis typical of PCOS associated with a hyperandrogenic pattern. These results support this hypothesis. In fact, androgen levels and clinical hyperandrogenism indices were lower in our patients with a type B ovary and partially in the hyperinsulinemic patients (Tables 3 and 4).

The hypothesis that different PCOS profiles have specific physiopathological pathways may have important implications for the management of patients and might imply a revision of the current PCOS diagnostic criteria. In other words, the attempt of the Rotterdam Consensus Group (Revised 2003 consensus, *Fertil Steril*, 2004) to define a shared, universal diagnosis of PCOS by gathering together patients with different characteristics and phenotypes may lead to a suboptimal approach for these patients even in fertility management. In detail, the insulinenic pattern seems to significantly influence ovarian response to gonadotropin administration (Ferorcsak *et al*., 2001) and ovarian drilling success (Amer *et al*., 2011). An increased risk of ovarian hyperstimulation syndrome in hyperinsulinemic patients has also been reported (Fulghesu *et al*., 1997). The insulin profile may also indirectly affect ovarian stimulation. In fact, insulin-sensitizing agents such as metformin before and during IVF/ICSI significantly reduced the incidence of ovarian hyperstimulation and improved the pregnancy rate (Tso *et al*., 2014). On the other hand, a hyperandrogenic profile may impair AMH production and lead to dysfunction of folliculogenesis (Eldar-Geva *et al*., 2005). The higher basal LH levels usually observed in hyperandrogenic women could also influence the ovarian response (Orvieto *et al*., 2012; Kumar *et al*., 2011). In detail, PCOS patients with an elevated LH/FSH ratio had a better pregnancy rate when treated with GnRH-agonist protocols than with GnRH-antagonist protocols, probably because the long GnRH-agonist induces prolonged LH suppression milieu thereby avoiding the negative effect of higher LH levels.
on reproductive outcome (Orvieto et al., 2012; Kumar et al., 2011; Homburg et al., 1988). Consequently, strategies that minimize the effect of LH could be considered for patients with a hyperandrogenic profile.

2.e Conclusion

This line of research has used PCOS as a model of impact of endogenous variables on the gonadal axis. The result of the association between insulin resistance and a peculiar ultrasound pattern, in patients affected, demonstrate the important role of this metabolic alteration on the pathology and so on the neuroendocrine axis function. Besides this observation, if confirmed by larger studies, supports the concept that specific PCOS profiles could be identified by a complete metabolic evaluation and targeted ultrasound pattern. Given the paucity of data regarding this issue, it remains to be seen if different PCOS profiles will lead to different diagnostic and eventually more tailored therapeutic approaches.
TAB 3 DEMOGRAPHIC, ANTHROPOMETRIC AND HORMONAL CHARACTERISTICS, AND ULTRASOUND PATTERN FREQUENCY IN THE STUDY POPULATION STRATIFIED ACCORDING TO INSULIN RESISTANCE.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Group 1</th>
<th>Group 2</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(n = 41)</td>
<td>(n = 32)</td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>28.6 ± 6</td>
<td>28.5 ± 4.8</td>
<td>0.93</td>
</tr>
<tr>
<td>BMI (kg/m$^2$)</td>
<td>34.5 ± 5.9</td>
<td>23.5 ± 2.5</td>
<td>0.0001</td>
</tr>
<tr>
<td>WHR</td>
<td>0.9 ± 0.5</td>
<td>0.8 ± 0.8</td>
<td>0.0001</td>
</tr>
<tr>
<td>HOMA</td>
<td>5.1 ± 2.9</td>
<td>1.5 ± 0.6</td>
<td>0.0001</td>
</tr>
<tr>
<td>Type B ovary</td>
<td>36/41 (87.8%)</td>
<td>7/32 (21.8%)</td>
<td>0.0001</td>
</tr>
<tr>
<td>Type A ovary</td>
<td>5/41 (12.1%)</td>
<td>25/32 (78.1%)</td>
<td>0.0001</td>
</tr>
<tr>
<td>Ovarian volume (cm$^3$)</td>
<td>14.6 ± 5.6</td>
<td>11.9 ± 4.1</td>
<td>0.024</td>
</tr>
<tr>
<td>SHBG (nmol/l)</td>
<td>27.2 ± 11.3</td>
<td>52.2 ± 21.1</td>
<td>0.0001</td>
</tr>
<tr>
<td>Ferriman-Gallwey score</td>
<td>10.7 ± 3.3</td>
<td>11.3 ± 3.2</td>
<td>0.47</td>
</tr>
<tr>
<td>Free testosterone (pg/ml)</td>
<td>2.5 ± 2.6</td>
<td>1.9 ± 1.5</td>
<td>0.36</td>
</tr>
<tr>
<td>DHEA-S (µg/dl)</td>
<td>282.1 ± 391.1</td>
<td>252.5 ± 304</td>
<td>0.74</td>
</tr>
<tr>
<td>17-OH-P (ng/ml)</td>
<td>1.2 ± 0.9</td>
<td>1.9 ± 1.7</td>
<td>0.93</td>
</tr>
</tbody>
</table>

Results reported as means ± SD or percentage (%)

BMI, body mass index; WHR, waist-height ratio; HOMA, homeostasis model assessment; SHBG, Sex hormone binding globulin; 17-OH-P, 17-hydroxy progesterone.
### Tab 4: Demographic, Anthropometric and Hormonal Characteristics of Patients Divided According to the Ultrasound Pattern of PCOS.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Type A ovary (n = 33)</th>
<th>Type B ovary (n = 45)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>28.4 ± 5.4</td>
<td>29 ± 5.8</td>
<td>0.61</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>23.7 ± 2.3</td>
<td>43.1 ± 56</td>
<td>0.048</td>
</tr>
<tr>
<td>WHR</td>
<td>0.9 ± 0.1</td>
<td>0.9 ± 0.1</td>
<td>0.0001</td>
</tr>
<tr>
<td>HOMA</td>
<td>1.5 ± 0.8</td>
<td>4.9 ± 2.8</td>
<td>0.0001</td>
</tr>
<tr>
<td>LH (UI/L)</td>
<td>9.25 ± 6.3</td>
<td>5.2 ± 3.3</td>
<td>0.002</td>
</tr>
<tr>
<td>Ovary volume (cm³)</td>
<td>12.1 ± 4</td>
<td>14.3 ± 5.6</td>
<td>0.048</td>
</tr>
<tr>
<td>SHBG (nmol/l)</td>
<td>53.5 ± 23.5</td>
<td>28 ± 11.6</td>
<td>0.0001</td>
</tr>
<tr>
<td>Ferriman-Gallwey</td>
<td>11.6 ± 2.9</td>
<td>10.5 ± 3.4</td>
<td>0.14</td>
</tr>
<tr>
<td>Free testosterone (pg/ml)</td>
<td>2.6 ± 2.6</td>
<td>2.1 ± 1.7</td>
<td>0.27</td>
</tr>
<tr>
<td>DHEA-S (µg/dl)</td>
<td>224.7 ± 221.7</td>
<td>164.4 ± 109.2</td>
<td>0.14</td>
</tr>
<tr>
<td>17-OH-P (ng/ml)</td>
<td>1.8 ± 1.5</td>
<td>1.2 ± 1.3</td>
<td>0.057</td>
</tr>
</tbody>
</table>

Results are reported as means ± SD

BMI, body mass index; WHR, waist-height ratio; HOMA, homeostasis model assessment; PCOM, polycystic ovarian morphology; SHBG, Sex hormone binding globulin; 17-OH-P, 17-hydroxy progesterone.
Fig. 2 Flow chart of patients enrolled in the study Standards for Reporting Diagnostic Accuracy (STARD).

Figure 1. Flow chart of patients enrolled in the study

Patients assessed for eligibility
\[ n = 309 \]

Patients not fulfilling the inclusion criteria
\[ n = 231 \]

Eligible participants
\[ n = 78 \]

Intermediate metabolic pattern
\[ n = 5 \]

Total for analysis
\[ n = 73 \]

Index test negative (No Type B ovary)
\[ n = 30 \]

Patients without insulin resistance
\[ n = 32 \]

Index test positive (Type B ovary)
\[ n = 43 \]

Patients with insulin resistance
\[ n = 41 \]
Fig. 3 Example of median ovarian section with the ovarian and stromal total areas defined. Calipers are positioned so as to encircle the total gonad circumference (A1) and the stromal component circumference (A2). The stroma/total area (S/A) was also calculated.

Fig. 4 Sonographic pattern of a type A ovary (A). Note the typical rosary arrangement of follicles and the easily recognizable hyperechogenicity that results from thickening of theca (B). The stromal hypervascularity is clearly visible in C. Secondary aspects are the dominance of follicles with a diameter > 4 mm and the predominance of longitudinal diameter (D).
Fig. 5 Sonographic pattern of a type B ovary (A). Note the ubiquitous arrangement of follicles, and the absence of central echogenicity (B). Characteristic signs of the type B ovary are a more "globular" gonad versus a type A ovary (C), with attenuation of the typical dominance of longitudinal diameter, and the presence of follicles with a mean diameter lower than those observed in Type A (D).
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Chapter II: The role of environmental factors: The Endometriosis model

1. Endometriosis

Endometriosis is considered one of the most frequent disease affecting the 6-10 % of women of reproductive age (2010).

It is characterized by the presence of endometrial-like tissue outside the uterus able to provoke a chronic inflammation of the pelvis. In rare circumstances, endometriosis could also involve extrapelvic organs. Clinical manifestations are very variable among patients and consist in dysmenorrhea, infertility, chronic pelvic pain, dyspareunia bowel and or urinary disorders when these organs are also involved.

1.a Etiology and pathogenesis

Several pathogenetic hypotheses have been advocated such as retrograde menstruation, lymphatic transport and coelomic metaplasiam, however the exact etiology still remains unknown (Sasson and Taylor, 2008), this is the reason why endometriosis is defined the “theories disease”.

Metastatic theory or retrograde menstruation

Samson (1927) was the first to suggest that the menstrual bleeding, containing fragments of endometrium, moves through the fallopian tubes in a retrograde way and then settles into the
peritoneal cavity. In order to verify this theory experimentally, endometriosis was induced by insertion of menstrual flow or endometrial tissue into the peritoneal cavity in rhesus monkeys. It has been reported that abnormalities of the genital tract and consequent construction of the leakage flow cause endometriosis in teenagers. The prevalence of retrograde menstrual bleeding was diagnosed in 90% of women laparoscopically, thus it was considered a physiological phenomenon. The fact that only 25% of women develops endometriosis suggests that retrograde menstruation is only the premise but not the cause of the disease. Besides this theory does not explain all the localizations of endometriosis such as the most distinct ones as the mediastinum and pleura.

**Celomic metaplasia**

Postulated by Meyer (1919), this theory held that endometriosis arises from original celomica membrane by a process of metaplasia as a result of prolonged irritation and/or estrogenic stimulation. A correlation between short cycles and stage of the disease was found. Celomic epithelium gives origin to the ovarian epithelial cells, to the Müllerian duct and to the peritoneal and pleural epithelium. Therefore, the mechanisms of this theory explain the presence of endometriosis in almost all the anatomical sites, including chest rectum and vaginal septum.

**Dissemination lymphatic and vascular**

Halban (1924) stated that endometrial cells once penetrated into lymphatic and blood vessels are responsible for an embolization process, often in ectopic sites. Although this mechanism can not be regarded as a common way of dissemination, however, it could be responsible for some rare extrapelvic localizations.

**Hormonal milieu**

It was suggested the hypothesis that endometriosis can depend on the presence of circulating steroid hormones. Receptors for estrogen, progesterone and androgens have been found in endometriosis deposits. The latter seems to be positively influenced by estrogen, while it undergoes atrophy when exposed to androgen stimulation.

**Immune system**

The relationship between endometriosis and immune response had been reported for the first time by Weed & Arquenborg in 1980. It’s plausible that the endometriotic disease is a consequence of
alterations in the immune system that promote the establishment of lesions in the peritoneal cavity after the retrograde menstruation process (Alviggi et al., 2009; Alviggi et al.; La Cava et al.; Matarese et al., 2003; De Placido et al., 2001; Matarese et al., 2000; De Placido et al., 1998; De Placido et al., 1994). Immunological studies have shown reduced activity of natural killer cells in women with endometriosis. Other studies showed an increased concentration of white blood cells (macrophages, natural killer cells and T lymphocytes) in the peritoneal fluid of women with endometriosis. The increased secretion of growth factors and cytokines suggests that the presence of endometriosis induces a local intrapelvic inflammatory reaction.

Independently of the different theories, the peritoneal environment seems to play an important role in pathogenesis. Molecular adhesion of cells and other factors produced by macrophages are responsible for the adhesion of fragments of endometrial tissue on the peritoneum. Later in the accession process, the growth of the endometrial tissue is favored and accelerated by steroid hormones, growth factors and angiogenic factors present in the peritoneal fluid.
2. The role of environmental pollutant in the pathogenesis of endometriosis: a systematic review of literature.

2.a Scientific background

The possible involvement of toxicants in the pathogenesis of endometriosis was proposed for the first time by Rier and collaborators in 1993. In detail, they observed a dramatically increase of severe endometriosis among rhesus monkeys colony exposed to 2,3,7,8 tetrachlorodibenzo-p-dioxin (TCDD) (Rier et al., 1993). This report, published more than 20 years ago, led to large number of investigations regarding the possible impact of environmental pollutant on the pathogenesis of endometriosis. Apart from dioxin like compound and TCDD, several toxic agent have been associated with endometriosis such as phthalic acids, non dioxin like polychlorinated biphenyls (PCB), bisphenol (BP), trace elements, heavy metals, perfluorochemicals (PFC), particulate matter and pesticides.

Fig.1 Adapted from Matarese et al. 2003.
Most of the published studies are retrospective and performed in small study populations. As consequence, contradictory results have been reported. In addition, methods adopted to quantify the effect were quite heterogeneous among studies. Hence, a definite conclusion about what kind of toxic agents are significantly associated with endometriosis was still not proposed. There are also scanty data about the underneath mechanisms by which environmental pollutants promote the growth of endometriosis.

A systematic review of literature was conducted with the aim to summarize all the available evidence regarding the relationship between environmental contaminants and endometriosis.

2.b Material and Method

Systematic research and strategy

A systematic research of the literature was performed with no time restriction. Only clinical trials and reviews involving human have been selected. Only paper in English, Italian, French and German was included.

Electronic searches were undertaken in the PUBMED, SCOPUS using the following keyword and search strategy: dioxins AND endometriosis; TCDD and endometriosis; PCB and endometriosis; dioxin like chemicals AND endometriosis; tetrachlorodibenzodioxin AND endometriosis; polychlorinated biphenyls AND endometriosis; phthalic acids AND endometriosis; phthalates AND endometriosis; cadmium and endometriosis; "Lead"[Mesh] AND endometriosis (PUBMED) OR “Lead” [Keyword] AND endometriosis (SCOPUS); heavy metals AND endometriosis; trace elements AND endometriosis; arsenic AND endometriosis; chromium AND endometriosis; mercury AND endometriosis; hydrocarbons, chlorinated AND endometriosis; toxic actions AND endometriosis; pesticides AND endometriosis; organochlorines pesticides and endometriosis; air pollutants AND endometriosis; air pollution AND endometriosis; particulate matter exposure AND endometriosis, particulate matter AND endometriosis; benzene AND endometriosis; waste management pollutants AND endometriosis; waste disposal facilities AND endometriosis; landfill AND endometriosis; waste management AND endometriosis; incineration AND endometriosis; incinerator AND endometriosis;

Moreover we hand-searched in the reference of selected journal article found through database search including article in press. All review selected was deeply analysed to detected every possible paper overlooked.
**Inclusion and exclusion criteria**

Inclusion criteria were all article and review regarding the association between endometriosis and environmental pollutants. Only peer reviewed articles were included. Only studies where the pollutant levels were directly measured in women have been included.

Exclusion criteria included: *in vitro* studies; Case report or case series; unpublished data; Study involving animals; the use of not conservative statistic method (Bayesian analysis). Studies with overlap between cases and controlled have also been excluded:

**Data extraction**

Studies and reviews were screened by 4 reviewers (A.C., R.B., C.B., F.D.) independently and any disagreement was solved by discussion. Firstly, all titles and abstracts from the databases were examined, but only those with the possibility of meeting the predefined criteria were kept for further evaluation. Final inclusion decisions were made on examination of the full manuscript. Hand searches of reference lists of literature review and *in press* article have been used to complement computer search.

**Quality assessment**

Newcastle Ottawa scoring system was adopted for the quality assessment of each study (Eliyahu *et al.*, 2016). Newcastle Ottawa scale allocates a maximum of 9 scores for case control and cohort studies and a maximum of 10 scores for cross-sectional study.

Specifically, each paper was independently evaluated by two authors (A.C and B.F) and final decision was reach after full discussion between authors. The following items were evaluated:

- Selection, comparability and exposure for case-control studies.
- Selection, comparability and outcome for cohort study and cross-sectional study.
2.c Results

**Dioxin-like compounds**

Belonging to the family of polyhalogenated aromatic hydrocarbons (PHAHs), dioxins-like compounds are a class of 210 organochlorines, divided into 75 polychlorinated dibenzo-p-dioxins (PCDDs) and 135 polychlorinated dibenzofurans PCDFs. On the other hand, PCBs dioxin like (PCB 77, 81, 105, 114, 118, 123, 126, 156, 157, 167, 169, 189) compounds also known as coplanar or non-ortho PCB, are twelve toxicants which show the same “dioxine-like” properties but could not be defined as dioxins (USA Environmental Protection Agencies). Among all dioxin products, 2,3,7,8 tetrachlorodibenzo-p-dioxin (TCDD) have demonstrated the most relevant toxic effect.

Dioxins are generally contaminants produced during industrial activities or waste incineration of chlorinated organic compounds. In addition, some amounts of dioxins are also produced during cigarettes smoking and steel manufactured process. Dioxins can also be generated by natural events, such as volcanic eruptions and forest fires. The most important exposure for humans mainly occurs by consumption of contaminated food (WHO, 2012) (WHO 2002).

Concentrations of dioxins are expressed as toxic equivalent factor (TEF) per g of lipids considering that adipose tissue constitute the main compartment store.

The majority of toxic activity for dioxin and dioxin like compounds is mediated by a specific receptor aryl hydrocarbon receptor (AhR) (Bruner-Tran and Osteen, 2010). The AhR is a ligand activated transcription factor which is present in cytosol as a complex associated with specific chaperone protein. When activated AhR is able to influence gene expression in the nucleus interacting with the aryl hydrocarbon nuclear translocator. The AhR is commonly expressed in the endometrium (Bruner-Tran and Osteen, 2010, Igarashi et al., 1999, Bulun et al., 2000) and in reproductive female tissue. It was also supposed to have relevant role in ovarian reserve (Richardson et al., 2014). AhR modulates metalloproteinases synthesis which is involved in endometriosis development (Tosti et al., 2015) and the usual resistance of ectopic endometrial cells to progesterone (Bruner-Tran et al., 1999).

Moreover, a strict interaction between estrogen receptor (ER) and AhR had been widely demonstrated (Richardson et al., 2014, Bruner-Tran and Osteen, 2010), supporting the fact that dioxin pollutant could significantly influence the function of estrogens and progesterone targets. An effect mediated by RANSES pathway was also demonstrated.
Finally, dioxins compounds are also able to interfere with immune-endocrine system of the endometrium enhanced the sensitivity to proinflammatory factors (Bruner-Tran et al., 2008).

A total of 20 studies has been included, 12 about dioxins and 8 about DLPCBs. Differences were found regarding the diagnostic method, type of pollutant analysed, type of controls, and method adopted for the assessment of the contaminants.

According to ESHRE guidelines, the gold standard diagnostic method consist in laparoscopic (or surgical) visualization and histological confirmation of endometriotic lesions, however the sole laparoscopy have a high negative predictive value (Dunselman et al., 2014). In the most of papers included (n = 18) endometriosis was assessed by laparoscopy and among these, in 9 out of 20, the diagnosis was confirmed by histology. A questionnaire was used by Fierens et al. In population study investigated by Eskenazi et al. 14 out of 19 cases was defined by laparoscopy or surgery.

Regarding controls selection, most of authors included patients where endometriosis had been excluded by laparoscopy. In Niskar et al. study (2009), only 30 out of 64 controls was determined by laparoscopy. On the other hand, Heiler et al (2004 and 2005) considered did not as control only patients with no clinical or ultrasound signs of endometriosis without laparoscopic or histologic confirmation.

In the most of papers dioxin compounds have been analysed with discordant results and diverse analytic methods. Dioxin compounds were measured in 12 studies (Mayani et al., 1997, Cai et al., 2011, De Felip et al., 2004, Eskenazi et al., 2002, Fierens et al., 2003, Heilier et al., 2005, Martinez-Zamora et al., 2015, Niskar et al., 2009, Pauwels et al., 2001, Porpora et al., 2009, Simsa et al., 2010, Tsuchiya et al., 2007)

Chemically activated luciferase expression bioassay (CALUX) assay was adopted in three studies (Pauwels et al., 2001, Porpora et al., 2009, Simsa et al., 2010) while in nine study a combination of gas chromatography and mass spectrometry was adopted for the measurement (Cai et al., 2011, De Felip et al., 2004, Eskenazi et al., 2002, Fierens et al., 2003, Heilier et al., 2005, Martinez-Zamora et al., 2015, Mayani et al., 1997, Niskar et al., 2009, Tsuchiya et al., 2007).

Results as well as type of pollutant analysed were also different among studies.

Regarding dioxin compounds the most of the studies did not found any significant association (Porpora et al., 2009, De Felip et al., 2004, Eskenazi et al., 2002, Fierens et al., 2003, Niskar et al., 2009, Pauwels et al., 2001, Tsuchiya et al., 2007) while five trials detected a possible significantly interaction (Cai et al., 2011, Heilier et al., 2005, Martinez-Zamora et al., 2015, Mayani et al., 1997,
Simsa et al., 2010). Specifically, both Heiler et al. in 2005 and Martinez Zamora et al. in 2014 founded a significant association with deep infiltrating endometriosis (Heilier et al., 2005, Martinez-Zamora et al., 2015).

In detail, Heiler et al. 2005 found a significantly increase of deep endometriotic nodules for an increment of 10 pg in total TEQ levels/g lipids of dioxin compounds (OR: 3.3; CI 95%, 1.4 –7.6) (Heilier et al., 2005). Recently, Martinez Zamora et al. compare dioxin like PCBs congener levels in omentum of patients affected by deep pelvic endometriosis with control without endometriosis. Toxic equivalence and concentration including all the pollutant analysed was found significantly higher in the omentum of the first group. Among all dioxins like PCBs analysed only PCB 114, 189, 126, 159 were statistically higher in DIE patients (Martinez-Zamora et al., 2015).

Cai et al. (2011) is the only study where dioxins levels were measured in peritoneal fluid. The authors founded higher concentration of PCDFs and DLPCBs in peritoneal fluid of women with endometriosis comparing with serum levels. Of interest, serum concentration of dioxin compounds was similar between case and controls. Finally, statistical results indicate that higher concentration of dioxin compounds in peritoneal fluid were associated with increased risk of endometriosis (OR 2.5 CI 95% 1.17-5.34; p = 0.035) (Cai et al., 2011).

Despite Porpora and Pauwels finding (Pauwels et al., 2001, Porpora et al., 2009) adopting the same method, Simsa et al. (2010) detected an increased risk to develop endometriosis among patients with high serum levels dioxin compound (≥ 25 pg TEQ/g lip) compared with lower level (≤14 og TEQ/g lip) (OR 2.44 CI 95% 1.4-5.70).

Mayani et al. (1997) and Eskenazi (2002) focused their attention only on the TCDD, who showed the most toxic properties among dioxin compounds. Specifically Mayani et al. (1997) designed the first case control where both cases and controls were ascertained with laparoscopy. Although a significant number of women positive for dioxin in serum was detected in endometriosis group (8/44 18% vs 1/35 3% p = 0.04) the OR did not reach statistical significance (OR: 7.6 CI 95% 0.91-169.7).

On the other hand Eskenazi et al. (2002) conducted large cohort trial in Seveso population almost 20 years later factory explosion in 1976 which resulted in elevated dispersion of dioxins compounds among rural population (Eskenazi et al., 2002). Population study was composed by 19 cases (14 surgical confirmed) and 277 controls (only 39 surgical confirmed). The authors observed a doubled but no significant relative risk ratio for women with higher TCDD levels >100ppt compared with patients with lower TCDD levels ≤20ppt (RR 2.1 CI 90% 0.5-8.0).
Polychlorinated biphenyls

Polychlorinated biphenyls were introduced in 1930 and are commonly used for their electrical insulating properties as dielectric fluids in transformers, capacitors and coolants. Belonging to the family of chlorinated hydrocarbons, these compounds are characterized by 1-10 chlorine atoms linked to a biphenyl in turn composed by the connection of two benzene rings by a carbon carbon bond (Bruner-Tran and Osteen, 2010). 209 congeners have been identified. The toxicity of these pollutants is strictly related to the number of chlorines.

Although their production was banned in 1976 these toxicants are still present in our environment including water and food. In addition there is evidence that exposure to PCBs could be associated with tumors like malignant melanoma, hepatocellular carcinoma and non Hodking lymphoma (Lauby-Secretan et al., 2015). All PCBs were classified as carcinogenic for humans, however the evidence is still considered inadequate in the human for toxicants with dioxin-like properties (Lauby-Secretan et al., 2015).

While dioxin-like PCBs exert their toxic effect through AhR, the role of non-dioxin like PCBs is still unclear. Some authors have reported a significant influence on the growth of endometrial cells to high exposure to PCBs (Johnson et al., 1997). Others have also postulated their interaction on pro-inflammatory activity and pro-metastatic factors (Sipka et al., 2008).

Furthermore, an antiestrogenic or estrogenic activity has been hypothesized among PCBs. One of the first characterization of estrogen activities of PCBs was proposed by Cooke in 2001 (Cooke PS, 2001) and used by Buck Louis et al. in 2005. Nonetheless, recent evidences are not consistent with these results (Table 2).

Among 21 studies which have investigated the role PCBs in the endometriosis, 17 studies focused about the possible role of NDL-PCBs congeners. The most investigated non dioxin like congeners were 138-153-180 (Table 1).

As for paper regarding dioxins compounds a relevant heterogeneity among studies was found.

In the most of paper selected endometriosis was assessed by laparoscopy (n = 18) and among these 9 with histological diagnosis. Only two studies adopted questionnaires to address endometriosis diagnosis.
Among all 209 congeners, only 45 PCBs congeners have been analysed (Table 3). Comparable levels of PCBs 18, 28, 44, 46, 74, 77, 81, 87, 99, 123, 128, 146, 149, 151, 157, 169, 172, 177, 178, 183, 187, 194, 195, 196, 201, 206, 209 was found both in patients and in controls.

On the other hand a significantly higher level in patients affected by endometriosis was found for seven DL PCBs congeners 105, 114, 118, 126, 156, 167, 189 and eleven NDL PCBs congeners 1, 5, 29, 52, 98, 101, 138, 153, 156, 170, 180. (Martinez-Zamora et al., 2015, Porpora et al., 2006, Porpora et al., 2009, Quaranta et al., 2006, Roya et al., 2009, Vichi et al., 2012, Gerhard and Runnebaum, 1992).

Specifically Quaranta et al (2006), Porpora et al. (2006), Porpora et al. (2009) and Vichi (2012) showed the same population study. Among these, the largest one (Porpora et al., 2009) reported a significant odds ratio for DL-PCB-118 OR = 3.79; 95% CI 1.61–8.91), NDL-PCB-138 (OR = 3.78; 95% CI, 1.60–8.94), NDL-PCB-153 (OR = 4.88; 95% CI, 2.01–11.0), NDL-PCB-170 (OR = 3.52; 95% CI, 1.41–8.79), NDL-PCB 180 OR 3.05 95% CI 1.25–7.42). The same results were no observed by the other authors with no significant odds ratio reported (Trabert et al., 2010; Niskar et al., 2009; Tsuchiya et al., 2007).

In a large matched cohort study, Buck Louis (2012) assesses the role of PCBs in two cohorts. The former constituted by women where the diagnosis of endometriosis was performed surgically (operative cohort), the latter where the endometriosis was diagnosed by MRI imaging (population cohort). All PCBs was measured both in serum and fat tissue in operative cohorts and only in serum in population cohort. Specifically, Buck Louis did no confirmed Porpora group findings both in population and operative cohorts: PCB 118 (Operative OR\textsubscript{a} 1.01 95% CI 0.83, 1.24; Population OR\textsubscript{a} 1.06 95% CI 0.66, 1.71); PCB 138 (Operative OR\textsubscript{a} 0.98 95% CI 0.80, 1.20 Population OR\textsubscript{a} 0.80 95% CI 0.40, 1.63); PCB 153 Operative OR\textsubscript{a} 0.87 95% CI 0.71, 1.07 Population OR\textsubscript{a} 1.13 95% CI 0.68, 1.89; PCB 170 (Operative OR\textsubscript{a} 0.79 95% CI 0.62 1.02 Population OR\textsubscript{a} 0.72 95% CI 0.32, 1.62); PCB 180 (Operative OR\textsubscript{a} 0.91 95% CI 0.74, 1.12 Population OR\textsubscript{a} 0.77 95% CI 0.40, 1.50) (Buck Louis et al., 2012). A reduced odds of endometriosis was reported for PCB 74 (OR\textsubscript{a} 0.72 95% CI 0.55-0.93) and PCB 156 (OR\textsubscript{a} 0.74 95% CI 0.57 0.96). All odds was adjusted for age, parity BMI, breastfeeding, serum cotinine levels and serum lipids. When breastfeeding an parity was removed and increased odds of endometriosis was observed for PCB 151 (OR\textsubscript{a} 3.23 95% CI 1.43-7.28).
Although several study showed higher levels of PCBs 1, 5, 29, 98, 128, 153,180 among patients with endometriosis no odds ratio was reported (Roya et al., 2009, Gerhard and Runnebaum, 1992, Reddy et al., 2006).

Of interest a possible association between PCBs and deep endometriosis was reported in a small size case control study. In detail several NDLPCBs (3, 8, 28, 52, 101, 153, 180, 194, 206, 209) and one DLPCB (118) have been assessed in serum of women with laparoscopic diagnosis of endometriosis and histologic diagnosis of adenomyosis. Comparing with controls, patients with adenomyosis showed higher levels of all PCBs analysed but no odds ratio was reported (Heilier et al., 2004).

In literature, there are scanty data regarding the relationship between NDL-PCBs congeners and deep endometriosis. Only one study form Heiler et al. in 2004 found an increased levels of total PCBs which include 10 NDL-PCBs congeners (3, 8, 28, 52, 101, 153, 180, 194, 206, and 209) and only one DL-PCBs congener (118) in patients with deep pelvic endometriosis (Heilier et al., 2004). The weight of the single NDL congeners was no reported by the authors.

**Trace elements**

Trace elements have long been studied in gynecology because of their interference with the endocrine system. The environmental presence of some of these, in particular cadmium, lead and mercury has been associated to the industrial activity and the presence of non-physiological detectable levels of these non-essential elements in the blood of most adults is to be considered a result of years of industrial activity and pollution. Adsorption of these substances is through the respiratory tract (inhalation) or through the digestive tract (ingestion of food, mainly fish and contaminated water). Through the bloodstream they reach various organs and systems with different mechanisms of action (Bridges and Zalups, 2005). Hormonal effects of these metals has been widely demonstrated by years of experience, until the identification of a specific group of emerging inorganic xenoestrogens, appropriately called metalloestrogens (Darbre, 2006). In particular lead and mercury, as bivalent metals, are capable to binding to the estrogen receptor (Zhang et al., 2008), by interfering with estrogentic activity and are thought to have antiestrogenic effects (Young et al., 1977; Martin, 2003); on the other hand cadmium has been shown to activate estrogen (ERalpha) and androgen receptor and inhibit the binding of Estradiol to ERalfa (Young et al., 1977, Stoica et al., 2000, Brama et al., 2007). Effects on female reproductive system are different, epidemiological studies have shown antiestrogenic effects of lead and estrogentic effects for
cadmium (Brama et al., 2007, Jackson et al., 2008, Silva et al., 2012) but is not still clear their role in several endocrine diseases.

To date few studies have evaluated relationship between this class of metalloestrogen and estrigenic diseases like endometriosis. Our analysis led us to the selection of four case-control studies (Heilier et al., 2004, Heilier et al., 2006, Silva et al., 2013, Turgut et al., 2013, Itoh et al., 2008) and one cohort study (Pollack et al., 2013). Among these we have excluded the study of Itoh (2008), since the control group included women with endometriosis stage I and II, which may be a confounding factor (Itoh et al., 2008).

The two studies of Heilier (2004, 2006) have not showed a role of cadmium in the pathogenesis of endometriosis, despite its demonstrated estrogenic activity, but showed significant lower levels of lead in the group with endometriosis. But it must emphasize that the two study groups did not differ in terms of age and smoking, but one of them (Heilier et al., 2006) differ in BMI; this may affect the proportion of endogenous estrogenic activity in the two groups. Moreover in both experiences while the diagnosis of endometriosis it was made through laparoscopy and histological confirmation, in the control group the presence of asymptomatic endometriosis cannot be excluded at all, not subjecting patients to laparoscopic examination.

In their experience, Silva et al. (2013) have demonstrated for the first time elevated blood levels of nickel in women with endometriosis, but this is an isolated result and would require further confirmation. In the same study group there were no different levels of lead and cadmium (Silva et al., 2013).

Turgut in 2013 found high levels of cooper in women with endometriosis compared to controls, but in the selection of patients has included among the cases of endometriosis only the advanced stages (III and IV). Also little is known about the possible role as endocrine disrupter of copper and for these reasons we cannot consider this result a clear link between exposure to copper and the development and progression of the disease (Turgut et al., 2013). The only cohort study (Pollack et al., 2013) does not clarify the relationship between cadmium, lead, mercury, arsenic, chromium and the pathogenesis of endometriosis, obtaining conflicting results in the different cohorts.

**Phthalates compound and endometriosis**

Phthalates compounds are widely used in plastic industries and added in considerably amounts to cosmetics and are considered as ubiquitous contaminants (Wittassek et al., 2011). Biochemically,
these compounds are characterized by alkyl diesters of phthalic acid which are rapidly metabolized and eliminated by urine in form of mono esters. For this reason urine analysis is considered a reliable tool to test phthalates exposure (Wittassek et al., 2011).

Considering the wide used of cosmetics among women, the exposure was estimated dramatically high in this population compared with men (Wittassek et al., 2011). In addition, phthalates compounds could be present in several medications such as didanosine, omeprazole and theophylline. As a matter of fact, in subjects taking these medications, a significantly higher levels of phthalates metabolites were observed (Hernandez-Diaz et al., 2009).

With respect of female reproductive activities, an involvement of phthalates in alteration of menstrual cycle and polycystic ovarian syndrome was suggested (Davis et al., 1994). In addition, an effect of phthalates products on reproduction was assessed in animal models (Agarwal et al., 1985, Agarwal et al., 1989). In vivo study demonstrated how some phthalates such as DEHP and DnBP could interact to estrogens receptors ESR 1 (Takeuchi et al., 2005).

The role of phthalates compounds in the pathogenesis of endometriosis were investigated. A total of nine studies have been identified in literature. In detail, an increased plasma levels of di-2 ethylhexyl phthalates (DEHP) was detected in 2003 in 35 endometriotic patients in a case control study (0.57 ug/ml versus 0.18 ug/ml; p = 0.0047) (Cobellis et al., 2003). Subsequently, a series of studies focused the role of phthalates compounds in the development of endometriosis in Indian population (Heilier et al., 2005, Reddy et al., 2006a, Reddy et al., 2006b, Rozati et al., 2008). Higher phthalates compounds levels concentrations were found in Indian patients affected by endometriosis with a progressively increasing in advanced stages. Huang et al. found higher levels of MnBP metabolites in the urine of women affected by endometriosis compared with control group. On the other hand, of seven urinary phthalates metabolites analysed (MMP, MEP, MnBP MBzP, MEHP, 5oxo-MEHP and 5OH-MEHP) no difference was seen in patient with adenomyosis (Huang et al., 2010).

Comparing the highest versus the lower of MBP and MEHP a large cross sectional study found a significant risk to develop both leiomyomatosis and endometriosis for urinary metabolites MBP (OR 1.71 CI 95% 1.07-2.75), but a weak inverse association for MEHP (OR 0.59 CI 95% 0.37 - 0.95) (Weuve et al., 2010). Nonetheless, both condition was self-reported by the patient with no surgical confirmation. In addition, when analysed alone no association found between all metabolites analysed and endometriosis. On contrary, elevated MEHP plasma levels in patients with
endometriosis was found by Kim et al. in 2011 in a case-control study of 266 women (97 cases and 169 controls) (OR 1.020 CI 95% 1.003–1.038; \( p = 0.020 \)) (Kim et al., 2011).

In 2013, Upson described for the first time an inverse relationship between endometriosis and MEHP urinary concentrations (adjusted OR 0.3 CI 95% 0.1, 0.7 \( p = 0.012 \)). The same authors detected a inverse no significant association with DEHP metabolites (5 OH MEHP and 5oxo MEHP). On the other hand their data showed and increased but no significant endometriosis risk with greater urinary concentrations of MBzP and MEP (Upson et al., 2013b).

On the other hand Buck Louis in the same years in a large cohort study described a 1.7 fold or higher odds of endometriosis for four phthalates compounds MECPP MEHP, 5 OH MEHP, 5oxo MEHP. When OR was adjusted for age creatinine and BMI also mBP and mCMHP resulted significantly associated with endometriosis. However, these findings was observed only in population cohort where endometriosis was diagnosed by magnetic resonance (MRI) (Buck Louis et al., 2013) Finally, the last study published in 2014 showed a only marginally increased levels of MEHP in patients affected by endometriosis or adenomyosis.

**Bisphenols**

The bisphenols are a group of chemical compounds with two hydroxyphenyl functionalities derived from the synthesis of plastics materials and some additives. "Bisphenol" is a common name, the letter following refers to one of the reactants. Bisphenol A (acetone) is the most popular representative of this group, often simply called "bisphenol". Bisphenols are endocrine disruptors and they have been characterized as a "pseudo-persistent" chemical leading to its spreading and potential accumulation in a variety of environmental matrices as the adipose tissue, giving rise to persistent, although low, serum levels (Yang et al Environ Health Perspect. 2011). Therefore, highly sensitive and selective analytical methods are needed for determination of low levels of BPA and BPB in serum samples. BPA shows estrogenic activity towards cell lines and endocrine-disrupting effects in vivo (Rier and Foster, 2002; Zeyneloglu et al., 1997; Cobellis et al., 2003; Ashby et al., 1998; Ashby and Odum, 2004; Bergeron et al., 1999; Kim et al., 2002; Matthews et al., 2001). The interaction of BPA and/or BPB with estrogenic receptor produces the activation of the same transcriptional-factor (CREB) as 17-\( \beta \)-estradiol (Tinwell et al., 2000; Quesada et al., 2002). Furthermore, previous studies have shown a relationship between BPA (and its metabolites) serum concentration increase and altered secretion of gonadotropic hormones and increase of androgenic hormones (Takeuchi et al., 2004, 2006). Probably, this mechanism determines a greater production
of estrogens, favoring the proliferative and inflammatory characteristics of endometriosis. The role of bisphenols compounds in the pathogenesis of endometriosis have been investigated. In our review a total of three studies have been included from literature. In one case-control study (Cobellis et al., 2009) the analyses of sera from both healthy and endometriotic women emphasized the absence of bisphenol in all the control cases (11 women), whereas BPA was found in 30 sera (51.7%) and BPB was found in 16 sera (27.6%) in the group of 58 patients with endometriosis; in nine of such sera BPA and BPB were present simultaneously. At least one of the bisphenols was found in 63.8% of sera of 58 patients with endometriosis. These findings strongly suggest the existence of a relationship between occurrence of endometriosis and the presence of BPA and/or BPB in the serum. One limitation of this study was the absence of multivariable analysis to adjust for potential confounders. In a matched cohort design “The ENDO Study” (M. Buck Louis et al., 2013), the aim was to assess the relation between persistent environment chemicals (phtalates and bisphenol a) and endometriosis by the hand of an operative and population cohort. The operative cohort comprised 495 women undergoing laparoscopy/laparotomy, while the population cohort comprised 131 women matched on age and residence. Surgically visualized or pelvic magnetic resonance imaging (MRI) diagnosed endometriosis in the two cohorts, respectively. The relation between BPA and endometriosis was evident in the population cohort (not in the operative one) and only emerged as significant when adjusting for parity along with other relevant covariates (AOR=1.97; 95% CI 1.04, 3.72). If endometriosis and parity share a common origin, its adjustment may induce over adjustment bias yielding a spurious finding. One study limitation includes the inability to detect endometriosis stages 1–2 in the population cohort, given the limited sensitivity and specificity of MRI for detecting milder disease relative to histologically confirmed disease. Despite errors associated with MRI diagnosed endometriosis relative to the clinical gold standard of visualization, the blinding of surgeons and radiologists to women’s chemical concentrations argues against biases.

**Organochlorine pesticides**

Organochlorine pesticides (OCPs) are widely used in the latter half of the 20th century. The OCPs are usually divided into three main groups including dichlorodiphenyltrichloroethane and its derivates (p,p’ DDT, p,p’-DDE, o,p’ DDT), isomers of hexachlorocyclohexane (β-γ HCH) and chlorinated cyclodiene such as aldrin and dieldrin. OCPs showed estrogenic activity in vitro (Andersen et al., 2002) and in animal model were able to alter normal reproductive functions (Alvarez et al., 2000, Shelby et al., 1996). A damage to central nervous system, liver, kidney and
bladder, derived from long term exposure to OCPs, was also observed (Shaw, 1992). Humans could be exposed to these pesticides via several routes including breathing polluted air, dermal penetration but contaminated food represent the main source of human exposure (Hassall, 1990). Limited attention has focused on persistent organochlorine pesticides (OCPs) and their association with endometriosis, despite their sharing a similar chemical structure with dioxins and PCBs and their ubiquitous presence in the environment. In our review we include 5 studies which investigate the association between endometriosis and OCPs exposure. Preliminary, data from Lebel and Gerrard did not reported any increased incidence of endometriosis among patients exposed to OCPs (Lebel et al., 1998, Gerhard and Runnebaum, 1992).

In one cohort study (Cooney et al. 2010), it has been found that OCPs were associated with an elevated adjusted odds of having a laparoscopically confirmed endometriosis diagnosis when OCP serum concentrations were in the highest tertiles for hexachlorobenzene (HCB) (aOR = 6.4; 95% CI, 1.0 - 42.8) compared with women in the lowest category. These associations persisted and were often stronger in the models when the concentrations of the chemicals were transformed and examined in a continuous fashion. The adjusted odds for endometriosis among women for a per unit log transformed increase in HCB was 1.4 (95% CI, 0.5, 3.9) and 5.0 (95% CI, 0.7 - 35.8) per unit log transformed increase in t-nonachlor. When OCP concentrations were left in their original unit but grouped by structure, significantly elevated aORs were observed for the highest tertile of aromatic fungicides (aOR = 5.3; 95% CI, 1.2 - 23.6) and elevated but not statistically significant aORs were observed for cyclodiene insecticides (aOR = 2.7; 95% CI, 0.8 - 9.5), as well as the midrange tertile for chlorinated insecticides (aOR = 1.6; 95% CI, 0.5 - 5.3). These findings all suggest an association between OCP exposure and endometriosis. Buck Louis (2012) in a large matched cohort study observed an significant association between endometriosis and HCH γ-β isomers respectively in operative (n = 473) and population cohort (n = 127) (γ HCH: adjusted OR = 1.27; 95% CI: 1.01, 1.59; β HCH adjusted OR = 1.72; 95% CI: 1.09, 2.72). Operative cohort and population cohort was constituted by women where endometriosis was diagnosed with laparoscopy and magnetic resonance respectively (Buck Louis et al., 2012).

In another population-based case-control study, OCPs were measured in serum of surgically confirmed endometriosis cases (n = 248) and from population-based controls (n = 538). An increased endometriosis risk associated with serum concentrations of β-HCH (third vs. lowest quartile: OR = 1.7; 95% CI: 1.0, 2.8; highest vs. lowest quartile OR = 1.3; 95% CI: 0.8, 2.4) and mirex (highest vs. lowest category: OR = 1.5; 95% CI: 1.0, 2.2). Interestingly, the association between serum β-HCH concentrations and endometriosis was stronger in patients with ovarian
endometriosis (third vs. lowest quartile: OR = 2.5; 95% CI: 1.5, 5.2; highest vs. lowest quartile: OR = 2.5; 95% CI: 1.1, 5.3) (Upson et al., 2013a).

**Perfluorochemicals**

Perfluorochemicals are organofluorine compounds containing carbon and fluorine characterized by long half-life (2.5 to 7.3 years) with elevated blood concentration documented in populations exposed (Olsen et al., 2007, Karrman et al., 2006); These man-made chemicals are used in several industrial applications: textile industries, cleaning aids and surfactants (Karrman et al., 2006). Diverse routes of exposure were suggested such as air, water, food and household dust, however the main one was not definitely established (Karrman et al., 2006).

Among persistent organic pollution only one study addressed the role of perfluorochemicals on endometriosis. In population where endometriosis was diagnosed by endoscopic visualization serum perfluorooctanoic acid (PFOA) (OR = 1.89 CI 95% = 1.17–3.06) and perfluorononanoic acid (OR = 2.20 CI 95% 1.02–4.75) was associated with endometriosis, matching both operative and population cohorts, perfluorooctane sulfonic (PFOS) acid (1.86 [1.05–3.30]) and perfluorooctanoic acid (PFOA)(OR = 2.58 CI 95 1.18–5.64) seemed to increase the odds for moderate/severe endometriosis (OR = 1.50 and 1.86, respectively). These findings were moderately attenuated with parity adjusted (Louis et al., 2012).

**Air pollution and particulate matter**

Air pollution consist in exposure to several kind of molecules including traffic exhaust (diesel and no diesel) including microscopic solid or liquid matter suspended in the earth atmosphere also knows as particulate matter (PM). Sources of PM are essentially man-made but could also be natural (volcanoes, seismic activities). They have impacts on climate and precipitation that adversely affect human health. PM with a diameter of 10 micrometers or less, also known as PM10, that can be inhaled but cannot cross the alveolar district. On the other hand, PM with a diameter of 2.5 micrometers or less, also known PM 2.5 can cross into the blood stream, deposit at distant tissues. Exposure to air pollution and PM is of great concerning in public health and were associated with increased mortality and hospitalization, especially for cardiovascular disease, stroke and lung disorders (Brook et al., 2010, Calderon-Garciduenas et al., 2011, Brook et al., 2004). Regarding the possible involvement in endometriosis pathogenesis, experiments in vivo have showed that in utero exposure to diesel exhaust could promote the persistence of endometriosis
lesion in rats (Umezawa et al., 2011). In addition there is evidence that exposure to air pollution may improve proinflammatory factors and oxidative (Brook et al., 2004, Brook et al., 2010) stress both involved in previous study in the development of endometriosis (Chen et al., 2010, Van Langendonekt et al., 2002).

There are few study which have address the role of particulate matter and air pollution on endometriosis pathogenesis.

We found one cohort study (Mahalingaiah et al., 2014) that evaluated the association of air pollution exposures during adulthood, including exposure to particulate matter (PM 2.5, PM 10-2.5, PM 10), and timing of exposure with risk of endometriosis in a cohort from the Nurses’ Health Study II with 10 years of follow-up. Only women who reported a laparoscopic diagnosis of endometriosis after 1993 through 2007 were included. Exposure to pollutant was evaluated by the calculation of the distance between residential address of participants and nearest road. For the primary analysis, the distance was categorized in 0-50 meters, 50-199 meters and ≥200 meters. Predicted ambient exposure to particulate was available using a data from the U.S. Environmental Protection Agency (EPA, 2013) Air Quality System, a nationwide network of continuous and filter-based monitors. The authors evaluated the hazard ratio of endometriosis by residential proximity to roadway and for each 10-μg/m3 increase in particulate matter. Finally, no significant ratio was reported for all PM analysed considering the exposure averaging time of 2 years, 4 years and cumulative. In addition, the authors found no significant hazard ratio to develop endometriosis with respect to distance to main roadway.
2.d Discussion

This line of research has pointed out the potential role of environmental pollutants in the pathogenesis of endometriosis. The neuro-ormonal control of the reproductive function is based on the connection of the hypothalamus-pituitary gland with the ovary, which in turn is crucial in controlling the competence of endometrium. So, it could be concluded that the endometrium is part of a whole which has been defined as hypothalamus-pituitary-gonadal-endometrial axis. Even if the etiology of endometriosis is still uncertain, the pathogenesis has two main steps: the ectopic distribution of the endometrial tissue and its response to the hormonal stimuli. The environmental pollutants seem to act as strong endocrine disruptors, causing and strengthening both the mechanism at the base of endometriosis, breaking the physiological connection between the hypothalamus-pituitary axis and the endometrial tissue. Even if this behavior has been studied for all the contaminants, through different mechanism as explained in the results, only few significant associations have been demonstrated.

Several studies supported the effect played by dioxins and PCBs on etiopathogenesis of endometriosis, so that it’s possible to conclude that there is a moderate evidence of the relation between endometriosis and these pollutants. In detail, higher levels of polychlorinated dibenzo-p-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs) and PCBs (especially PCBs 138, 153, 180) were observed in patients affected by endometriosis. There are also evidence that support a role of PCBs and dioxins in the pathogenesis of deep endometriosis and adenomyosis. Contrasting results were found regarding the association between phthalates and endometriosis. With respect of organochlorines pesticides, only β-hexachlorocyclohexane was associated with an increased odds of endometriosis. Only one study reported a significantly associations between perfluorochemicals [perfluorooctanoic acid (PFOA) and perfluorononanoic (PFNA)] and benzophenone metabolites [2,4-dihydroxybenzophenone (2OH BP)] with endometriosis. No association was found between endometriosis and trace elements, including cadmium, despite its demonstrated estrogenic activity. This can be explained by the presence of a endogen protect mechanism from the toxic effects of heavy metals, such the metallothionine (Klaassen CD, J Toxixol Sci, 1998; Waalker HP, Cancer Research, 2004). To date, a direct relation between trace elements and endometriosis cannot be demonstrated.

This study has some limitations that couldn’t be overcome and that could have interfered on the analysis. First, the measurement used to estimate the personal exposures is indirect as it is based on the ambient exposure and not always on blood analysis. Second there are no information on
workplace exposures or the proportion of days spent at home, or on domestic characteristics (e.g.,
age, ventilation rate, air purification systems) that may influence the levels of ambient pollution
exposure inside the home; these factors can all lead to an exposure misclassification. Another
limitation is that the exposure is valued only during adulthood not considering childhood and
intrauterine environment. This time window could not be at the most etiologically relevant in
relation to endometriosis disease pathogenesis.

2.e Conclusion

This line of research has partially confirmed the role of endocrine disruptor of environmental
pollutants on the gonadal axis. In fact, based on the available data obtained, an association between
endometriosis and specific environmental pollutants cannot be excluded, even if at moderate and
low level of evidence. In literature, this is the only study that tried to verify an association between
endometriosis and air pollution-particulate matter exposure so larger trials and well conducted
studies are demanding.
Fig. 2a Detailed flow chart 1.

Original queries (n = 627)
Scopus = 330
Pubmed = 327

Duplication of titles (n = 178)
Excluded on first pass (n = 337)

Potentially relevant abstracts (n = 142)
Scopus = 71
Pubmed = 71

Excluded on second pass (n = 25)
Duplication of data = 2
Overlap cases and controls = 4
No conservative statistic methods = 3
Language restriction = 3
Unpublished data = 1
Pollutant no directly measured = 5
Others = 6

Eligible trials included in the systematic review (n = 46)
Dioxins-like compounds and PCBs: n = 19
Pthalathes: n = 9
PCBs + pthalathes: n = 1
Organochlorine pesticides: 2
Organochlorine pesticides + Dioxins/PCBs = 5
Trace elements: n = 5
Perflorochemicals: n = 1
Benzophenone: n = 1
Particulate matter: n = 1
Bisphenols n = 2
Fig. 2b Flow chart according PRISMA statement.

Records identified through database searching (n = 657)

Additional records identified through other sources (n = 2)

Records after duplicates removed (n = 481)

Records screened (n = 481)

Records excluded (n = 339)

Full-text articles assessed for eligibility (n = 71)

Full-text articles excluded, with reasons (n = 25)

Studies included in qualitative synthesis (n = 46)
TAB 1.
Summary of study examining the involvement of environmental pollutant on the pathogenesis of endometriosis. The paper has been classified according the type of pollutant involved.

<table>
<thead>
<tr>
<th>Pollutant</th>
<th>Author, Year, (ref)</th>
<th>Study design</th>
<th>Population</th>
<th>Diagnosis method</th>
<th>ON Score</th>
<th>Method (pollutant analysis)</th>
<th>Exposure</th>
<th>Exposure evaluation</th>
<th>Confounders adjusted for</th>
<th>Results: effect estimation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dioxins and PCB</td>
<td>Martinez-Zamora et al. 2015</td>
<td>Case control</td>
<td>Spain</td>
<td>Laparoscopy and histology (DEND)</td>
<td>7</td>
<td>HR-GC/HR-MS</td>
<td>Omentum</td>
<td></td>
<td>Breastfeeding</td>
<td>2,3,4,7,8-PeCDF, 2,3,7,8-TCDD, 1,2,3,7,8-PeCDD were significantly higher in patients with DIE</td>
</tr>
</tbody>
</table>
|                    | Buck Louis et al. 2012            | Matched Cohort | USA        | Operative cohort: 190 cases 283 controls
Population cohort: 14 cases
Laparoscopy and histology (operative cohort)
Magnetic resonance (population cohort) | 8        | GC/HRMS electronic capture detector | Serum Omentum |                       | Age BMI Breastfeeding Lipids Codipine | Crude OR significant for PCB 28,151,201
PCB 153 showed a significant OR when breastfeeding was removed from the model |

Country Individuals

ON Score

Method

Exposure evaluation

Confounders adjusted for

Results: effect estimation
<table>
<thead>
<tr>
<th>Study</th>
<th>Design</th>
<th>Country</th>
<th>Cases</th>
<th>Controls</th>
<th>Diagnostic Methods</th>
<th>Analysis Methods</th>
<th>PCBs Identified</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vichi et al. 2012</td>
<td>Case</td>
<td>Italy</td>
<td>63</td>
<td>63</td>
<td>Laparoscopy and histology</td>
<td>HR-GC/Ion trap MS</td>
<td>NDL PCB: 28, 52, 101, 138, 153, 170, 180 DL PCB 105, 118, 156, and 167.</td>
<td>Lipid breastfeeding and Lipid</td>
</tr>
<tr>
<td>Cai et al. 2011</td>
<td>Case</td>
<td>Japan</td>
<td>10</td>
<td>7</td>
<td>Laparoscopy</td>
<td>GC/HR-MS</td>
<td>Dioxin compounds: 7 PCDDs; 10 PCDF and all dioxin like PCBs</td>
<td>Lipid</td>
</tr>
<tr>
<td>Trabert et al. 2010</td>
<td>Case</td>
<td>USA</td>
<td>251</td>
<td>538</td>
<td>Laparoscopy (cases) Interview (controls)</td>
<td>HR-GC/HR-MS</td>
<td>18 NDLPCBs: 18, 28, 44, 49, 52, 66, 74, 99, 138, 153, 170, 180, 187, 194, 196, 201, 206, and 209 2 DLPCBa: (118, 156)</td>
<td>Lipid</td>
</tr>
<tr>
<td>Sisma et al. 2010</td>
<td>Case</td>
<td>Belgium</td>
<td>96</td>
<td>105</td>
<td>Laparoscopy and histology</td>
<td>CALUX</td>
<td>Dioxin compounds</td>
<td>Lipid</td>
</tr>
<tr>
<td>Study</td>
<td>Study Type</td>
<td>Location</td>
<td>Cases/Controls</td>
<td>Methodology</td>
<td>Analysis</td>
<td>PCBs</td>
<td>Significant Findings</td>
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<td></td>
</tr>
<tr>
<td>Roya et al. 2009</td>
<td>Case control</td>
<td>India</td>
<td>86 cases/91 controls</td>
<td>Laparoscopy</td>
<td>5</td>
<td>GC flame ionization detector</td>
<td>PCB 1, 5, 29, 98</td>
<td>Significant increase in endometriosis group, related with stage</td>
</tr>
<tr>
<td>Porpora et al. 2009</td>
<td>Case control</td>
<td>Italy</td>
<td>80 cases/78 controls</td>
<td>Laparoscopy and histology</td>
<td>8</td>
<td>CALUX HRGC/MS ion trap</td>
<td>DLPCBs (105,118,156,167) NDLPCBs (28,52,101,138,153,167, 170-180) Dioxin compounds; Age, smoking habits, BMI, Weight changes</td>
<td></td>
</tr>
<tr>
<td>Niskar et al. 2009</td>
<td>Case control</td>
<td>USA</td>
<td>60 cases/64 controls</td>
<td>Laparoscopy and histology</td>
<td>8</td>
<td>HRGC/HRMS</td>
<td>3 PCDD; 2 PCDF; DLPCBs (118,126, 156,169); NDLPCBs (138,153,180); ppDDE; Lipid analysis and breast feeding</td>
<td>No significant differences</td>
</tr>
<tr>
<td>Tsuchiya et al. 2007</td>
<td>Case control</td>
<td>Japan</td>
<td>79 cases (31 stage I-II) (48 stage III IV) 59 controls</td>
<td>Laparoscopy</td>
<td>7</td>
<td>GS/HRMS</td>
<td>8 PCDDs 10 PCDFs 4 DL PCBs 36 NDLPCBs</td>
<td>Lipid analysis</td>
</tr>
<tr>
<td>Hoffman et al., 2007</td>
<td>Cohort USA</td>
<td>79 cases 864 controls</td>
<td>Questionnaire</td>
<td>GC/ electron capture detection</td>
<td>Serum analysis</td>
<td>PBBs PCBs</td>
<td>Age Household income, Menopausal status, PBB levels</td>
<td>Increased incidence of endometriosis among women exposed to moderate PCB and high PCB</td>
</tr>
<tr>
<td>Study</td>
<td>Design</td>
<td>Country</td>
<td>Cases/Controls</td>
<td>Procedure</td>
<td>Analytical Method</td>
<td>PCB Analysis</td>
<td>PCB Level</td>
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<tr>
<td>Porpora et al., 2006</td>
<td>Case control</td>
<td>Italy</td>
<td>40 cases / 40 controls</td>
<td>Laparoscopy</td>
<td>HRGC/MS ion trap</td>
<td>Serum analysis</td>
<td>2 DLPCB (118, 156) 6 NDL (28, 52, 101, 138, 153, 180) metabolic disease, gravidity, parity, weight changes</td>
<td></td>
</tr>
<tr>
<td>Quaranta et al., 2006</td>
<td>Case control</td>
<td>Italy</td>
<td>10 cases / 8 controls</td>
<td>Laparoscopy</td>
<td>HRGC/MS ion trap</td>
<td>Serum analysis</td>
<td>1 DLPCB (118) 2 NDLPCB (138,153) p,p'-DDE Significant increase serum concentration of PCB 118,138, 153 and p,p'-DDE in patients with endometriosis</td>
<td></td>
</tr>
<tr>
<td>Reddy et al., 2006</td>
<td>Case control</td>
<td>India</td>
<td>85 cases / 135 controls</td>
<td>Laparoscopy</td>
<td>GC</td>
<td>Plasma analysis</td>
<td>PCB: 1, 5, 29; 98 (no coplanar) Borderline significance for anti-estrogenic PCBs (p: 0.08) that are lower in endometriosis; no differences for estrogenic PCBs</td>
<td></td>
</tr>
<tr>
<td>Buck Louis et al., 2005</td>
<td>Case control</td>
<td>USA</td>
<td>32 cases / 52 controls</td>
<td>Laparoscopy</td>
<td>GC/electron capture detection</td>
<td>Serum analysis</td>
<td>7 DLPCBs (105, 114, 118, 126, 167, 169, 189) 55 NDLPC Dioxin like PCBs were significantly higher in adenomyosis; PCDD/PCDF were significantly higher in cases than controls; significantly increase risk of adenomyosis and endometriosis</td>
<td></td>
</tr>
<tr>
<td>Heilier et al., 2005</td>
<td>Case control</td>
<td>Belgium</td>
<td>25 END / 25 DEND / 21 controls</td>
<td>END:laparoscopy DEND:histology</td>
<td>GC/HR-MS</td>
<td>Serum analysis</td>
<td>17 PCDD/Fs, 12 dioxin like PCBs standardization for BMI and age</td>
<td></td>
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<tr>
<td>Heilier et al., 2004</td>
<td>Case control</td>
<td>Belgium</td>
<td>10 DEND / 7 END / 10 controls</td>
<td>END:laparoscopy DEND:histology</td>
<td>GC/HR-MS</td>
<td>Serum analysis</td>
<td>DLPCBs (118) NDLPCBs (3, 8, 28, 52, 101, 153, 180, 194, 206, 209) Age serum levels of all PCBs analysed significantly higher in patients with adenomyosis</td>
<td></td>
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<tr>
<td>Study</td>
<td>Study Type</td>
<td>Country</td>
<td>Cases/Controls</td>
<td>Methodology</td>
<td>Analytical Technique</td>
<td>Analysis</td>
<td>Outcome</td>
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<tr>
<td>De Felip et al. 2004</td>
<td>Case/Control</td>
<td>Italy/Belgium</td>
<td>23 cases/17 controls</td>
<td>Laparoscopy and histology</td>
<td>GC-HRMS</td>
<td>Serum analysis</td>
<td>17 PCDD/PCDFs, 12 dioxin-like PCBs</td>
<td></td>
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<tr>
<td>Fierens et al. 2003</td>
<td>Case/Control</td>
<td>Belgium</td>
<td>10 cases/132 controls</td>
<td>Questionnaire</td>
<td>GC-HRMS</td>
<td>Serum analysis</td>
<td>17 PCDD/Fs, 11 NDLPCBs (3,8,28,52,10,138,153,180,194,206,209), 5 DLPCBs (77, 81, 118,126)</td>
<td></td>
</tr>
<tr>
<td>Eskkenazi et al. 2002</td>
<td>Cohort</td>
<td>Italy</td>
<td>19 cases/277 controls (14 surg cases/39 surg contr.)</td>
<td>Laparoscopy no in all cases</td>
<td>GC/HRMS</td>
<td>Serum analysis</td>
<td>No differences between groups in terms of PCDD/Fs and coplanar PCBs d PCBs in cases and controls; 12 PCB markers were slightly lower in endometriosis not significant: RRR 1.1 (90% CI 0.2-7.8) in group 20-100 ppt; RRR 3.6 (90% CI 0.5-27.0) in &gt;100 ppt doubled not significant risk for endometriosis among woman with TCDD &gt;100ppt.</td>
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</tr>
<tr>
<td>Pauwels et al. 2001</td>
<td>Case/Control</td>
<td>Belgium</td>
<td>42 cases/27 controls</td>
<td>Laparoscopy</td>
<td>CALUX GC-ECD</td>
<td>Serum analysis</td>
<td>Dioxins compounds 3 NDLPCBs (138,153,180)</td>
<td></td>
</tr>
<tr>
<td>Lebel et al. 1998</td>
<td>Case/Control</td>
<td>Canada</td>
<td>86 cases/70 controls</td>
<td>Laparoscopy</td>
<td>GC/ECD</td>
<td>Plasma analysis</td>
<td>BMI Alcohol intake</td>
<td></td>
</tr>
</tbody>
</table>

No significant difference in cases and controls; lower body burden levels in Italian population no differences between groups in terms of PCDD/Fs and coplanar PCBs d PCBs in cases and controls; 12 PCB markers were slightly lower in endometriosis no significant: RRR 1.1 (90% CI 0.2-7.8) in group 20-100 ppt; RRR 3.6 (90% CI 0.5-27.0) in >100 ppt doubled not significant risk for endometriosis among woman with TCDD >100ppt. No statistical differences No statistical differences
<table>
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<tr>
<th>Study</th>
<th>Design</th>
<th>Country</th>
<th>Cases/Controls</th>
<th>Diagnostic Method</th>
<th>Analysis Method</th>
<th>Pollutants</th>
<th>Odds Ratio/Significance</th>
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</thead>
<tbody>
<tr>
<td>Mayani et al. 1997</td>
<td>Case control</td>
<td>Israel</td>
<td>44/35 cases</td>
<td>Laparoscopy</td>
<td>GC/MS</td>
<td>Blood analysis TCDD</td>
<td>Non significant OR 7.6</td>
</tr>
<tr>
<td>Gerhard and Runnebaum 1992</td>
<td>Case control</td>
<td>Germany</td>
<td>28/441 cases</td>
<td>Laparoscopy</td>
<td>n.a.</td>
<td>Blood analysis NDL-PCB 128,153,180</td>
<td>PCB significantly higher among endometriosis group</td>
</tr>
<tr>
<td>Huang et al. 2014</td>
<td>Case control</td>
<td>Taiwan</td>
<td>44 (16 adeno) / 69 controls</td>
<td>Laparoscopy and histology</td>
<td>LC/Electrospray ionization MS</td>
<td>Urinary phthalates metabolites MMP, MEP, MnBP, MBzP, MEHP, 5oxo-MEH, SOH-MEH</td>
<td>Endometriosis/adenomyosis women showed a marginally increased level of urinary MEHP only</td>
</tr>
<tr>
<td>Buck Louis et al. 2013</td>
<td>Matched cohort</td>
<td>USA</td>
<td>190/283 cases</td>
<td>Laparoscopy/Surgery and histology (operative cohort) MRI (population cohort)</td>
<td>Enzymatic deconjugation and solid phase extraction</td>
<td>Urinary phthalate metabolites MECPP, MCMHP, 5oxo-MEH, 5OH-MEH, MEHP, MCPP, MMP, MEP, MiBP, MnBP, MCHP, MBzP, MNP, MOP, MBP BPA</td>
<td>Operative cohort no difference for all pollutant analysed Population cohort: MECPP MEHP, 5 OH MEHP, 5 oxo MEHP: 1.7 fold or higher odds of endometriosis</td>
</tr>
<tr>
<td>Study</td>
<td>Type</td>
<td>Country</td>
<td>Cases</td>
<td>Controls</td>
<td>Methodology</td>
<td>Metabolites</td>
<td>Analysis</td>
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<tr>
<td>Upson et al. 2013</td>
<td>Case control</td>
<td>USA</td>
<td>92</td>
<td>195</td>
<td>Laparoscopy/ Surgery</td>
<td>MEHP, 5OH-MEHP, 5oxo-MEHP, MECPP, MBzP, MEP, MiBP, MnBP</td>
<td>LC-MS</td>
</tr>
<tr>
<td>Kim et al. 2011</td>
<td>Case control</td>
<td>Korea</td>
<td>97</td>
<td>169</td>
<td>Surgical and histology</td>
<td>MEHP, DEHP</td>
<td>LC-MS</td>
</tr>
<tr>
<td>Weuve et al. 2010</td>
<td>Cross-sectional</td>
<td>USA</td>
<td>1020</td>
<td>87</td>
<td>Questionnaire</td>
<td>MEHP, MBP, MEP, MBzP, 5OH-MEHP, 5oxo-MEHP</td>
<td>LC-MS</td>
</tr>
<tr>
<td>Huang et al. 2010</td>
<td>Case control</td>
<td>Taiwan</td>
<td>29</td>
<td>29</td>
<td>Surgery and histology</td>
<td>MMP, MEP, MnBP MBzP, MEHP, 5oxo-MEHP and 5OH-MEHP</td>
<td>LC-MS</td>
</tr>
<tr>
<td>Roya et al. 2008</td>
<td>Case control</td>
<td>India</td>
<td>99</td>
<td>135</td>
<td>Laparoscopy</td>
<td>DMP, DEP, DeBP BBP, BEHP</td>
<td>HP-LC</td>
</tr>
</tbody>
</table>

**Notes:**
- MEHP: Monoethylhexyl phthalate
- 5OH-MEHP: 5-OH-monoethylhexyl phthalate
- 5oxo-MEHP: 5-oxo-Monoethylhexyl phthalate
- MBzP: Monobenzyl phthalate
- MEP: Monooctyl phthalate
- MiBP: Methyl-2-butoxy phthalate
- MnBP: Methyl-nonyl phthalate
- MMP: Mono-2-methylphthalate
- MBP: Mono-2-butoxy phthalate
- 5OH-MEHP: 5-OH-Monoethylhexyl phthalate

**Inverse associations:**
- MEHP (4th vs 1st quartiles (aOR 0.3, 95% CI: 0.1–0.7)
- MEHP Elevated in endometriosis women OR 1.020 (CI 95%: 1.003–1.038, p = 0.20)

**Positive associations:**
- MBP and endometriosis or leiomyomatosis and weak inverse association of MEHP and endometriosis or leiomyomatosis
- Creatinine adjusted MnBP higher in cases
<table>
<thead>
<tr>
<th>Study</th>
<th>Type</th>
<th>Country</th>
<th>Cases</th>
<th>Controls</th>
<th>Procedure</th>
<th>Assays</th>
<th>Methodology</th>
<th>Summary</th>
</tr>
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<tbody>
<tr>
<td>Reddy et al. 2006</td>
<td>Case</td>
<td>India</td>
<td>49</td>
<td>38</td>
<td>Laparoscopy</td>
<td>6</td>
<td>HP-LC GC</td>
<td>Plasma analysis of DnBP, BBP, DnOP, DEHP higher in endometriosis groups and significantly correlated with endometriosis severity</td>
</tr>
<tr>
<td>Reddy et al. 2006</td>
<td>Case</td>
<td>India</td>
<td>85</td>
<td>135</td>
<td>Laparoscopy</td>
<td>7</td>
<td>GC</td>
<td>Plasma analysis of DnBP, DEHP, DnOP, BBP. Phthalates esters higher in cases Higher value of both pollutant in advanced stage.</td>
</tr>
<tr>
<td>Cobelli et al. 2003</td>
<td>Case</td>
<td>Italy</td>
<td>35</td>
<td>24</td>
<td>Laparoscopy</td>
<td>7</td>
<td>HP-LC</td>
<td>Serum Peritoneal fluid analysis of DEHP MEHP Significantly higher serum levels of DEHP in patients with endometriosis</td>
</tr>
</tbody>
</table>

**Organochlorine pesticides**

- β-hexachlorocyclohexane (β-HCH), γ-hexachlorocyclohexane (γ-HCH), heptachlor epoxide, oxychlordane, trans-nonachlor, two isomers of dichlorodiphenyltrichloroethane (p,p'-DDT, o,p'-DDT), dichlorodiphenyldichloroethylene (p,p'-DDE), dieldrin, hexachlorobenzene, Age, Reference date, Year, Smoking, Alcohol, Education, Total lipids, Ethnicity. β-HCH and mirex associated with endometriosis. Stronger association between β-HCH and in subgroups affected by ovarian endometriosis.
<table>
<thead>
<tr>
<th>Study</th>
<th>Design</th>
<th>Country</th>
<th>Operative cohort:</th>
<th>Population cohort:</th>
<th>Methods</th>
<th>Measure</th>
<th>Results</th>
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<tbody>
<tr>
<td>Buck Louis et al. 2012</td>
<td>Matched</td>
<td>USA</td>
<td>190 cases 283</td>
<td>14 cases 113</td>
<td>Operative: Laparoscopy/ Surgery and histology (operative cohort) Population: Magnetic resonance (population cohort)</td>
<td>OCPs: HCB, HCH, γ-HCH, β-HCH, oxychlordane, cis- and trans-nonachlor, cis- and trans-chlordane, p,p'-DDT p,p'-DDE α,ω-DDE</td>
<td>γ-HCH associated with endometriosis in operative cohort β-HCH associated with endometriosis in population cohort</td>
</tr>
<tr>
<td>Cooney et al. 2010</td>
<td>Case</td>
<td>USA</td>
<td>29 cases 51</td>
<td></td>
<td>Laparoscopy</td>
<td>Serum analysis</td>
<td>HCB associated with increased odds of endometriosis</td>
</tr>
<tr>
<td>Porpora et al. 2009</td>
<td>Case</td>
<td>Italy</td>
<td>80 cases 78</td>
<td></td>
<td>Laparoscopy and histology</td>
<td>Serum analysis</td>
<td>Age, smoking habits, BMI, Weight changes</td>
</tr>
<tr>
<td>Quaranta et al. 2006</td>
<td>Case</td>
<td>Italy</td>
<td>10 cases 8</td>
<td></td>
<td>Laparoscopy and histology</td>
<td>Serum analysis</td>
<td>Significant increase serum concentration of p,p'-DDE in patients with endometriosis</td>
</tr>
<tr>
<td>Lebel et al. 1998</td>
<td>Case</td>
<td>Canada</td>
<td>86 cases 70</td>
<td></td>
<td>Laparoscopy</td>
<td>Plasma analysis</td>
<td>Age BMI</td>
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<table>
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<tr>
<th>Study</th>
<th>Design</th>
<th>Country</th>
<th>Cases</th>
<th>Controls</th>
<th>Procedure</th>
<th>Blood Analysis</th>
<th>Trace Elements</th>
<th>Findings</th>
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<tbody>
<tr>
<td>Gerhard and Runnebaum 1992</td>
<td>Case control</td>
<td>Germany</td>
<td>28 cases</td>
<td>441 control</td>
<td>Laparoscopy/Surgery</td>
<td>n.a.</td>
<td>Blood analysis</td>
<td>HCH, HCB, DDT, DDE, DDD</td>
</tr>
<tr>
<td>Turgut et al. 2013</td>
<td>Case control</td>
<td>Turkey</td>
<td>31 cases</td>
<td>41 controls</td>
<td>Laparoscopy/surgery and histology</td>
<td>5</td>
<td>Copper: Spectrometry</td>
<td>Serum analysis</td>
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<tr>
<td>Silva et al. 2013</td>
<td>Case control</td>
<td>Sri Lanka</td>
<td>50 cases</td>
<td>50 controls</td>
<td>Laparoscopy/Surgery</td>
<td>5</td>
<td>The total-reflection X-ray fluorescence</td>
<td>Blood analysis</td>
</tr>
<tr>
<td>Pollack et al. 2013</td>
<td>Matched cohort</td>
<td>USA</td>
<td>Operative cohort: 190 cases</td>
<td>283 controls</td>
<td>Laparoscopy/Surgery and histology (operative cohort)</td>
<td>MRI (population cohort)</td>
<td>Blood and urine analysis</td>
<td>Urine: antimony, arsenic (As), barium, beryllium, cadmium, cesium, chromium, cobalt (Co), copper, lead, manganese, mercury, Age, BMI, Smoking, Race, Vitamin use Creatinine, Parity/gravidity</td>
</tr>
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Cu (μg/ml): 1088.00 ± 273.58 vs 811.20 ± 265.77 < 0.001
<table>
<thead>
<tr>
<th>Study</th>
<th>Design</th>
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<th>Cohort Size</th>
<th>Methodology</th>
<th>Outcome</th>
<th>Risk Factor</th>
<th>Findings</th>
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<tbody>
<tr>
<td>Heilier et al. 2006</td>
<td>Case control</td>
<td>Belgium</td>
<td>119 cases 25 controls</td>
<td>Laparoscopy 5</td>
<td>Serum and urine analysis</td>
<td>cadmium, lead</td>
<td>No correlation for cadmium; lower serum levels of lead in endometriosis</td>
</tr>
<tr>
<td>Heilier et al. 2004</td>
<td>Case control</td>
<td>Belgium</td>
<td>38 cases (25 endometriosis) 21 controls</td>
<td>Laparoscopy in cases, controls clinical exam 7</td>
<td>Serum and urine analysis</td>
<td>cadmium</td>
<td>No difference in serum among the groups; in urine, not difference significant, but the mean was slightly higher in adenomyosis</td>
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<td>Perfluorochemicals</td>
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</tr>
<tr>
<td>Buck Louis et al. 2012</td>
<td>Cohort</td>
<td>USA</td>
<td>190 cases 283 controls</td>
<td>Laparoscopy and histology (operative cohort)</td>
<td>Serum analysis</td>
<td>PFDA, PFHxS, PFNA, PFOA, PFOS, PFDoDA, PFHpA, PFOSA, PFUnDA</td>
<td>Serum perfluorooctanoic acid and perfluorononanoic acid associated with endometriosis in operative cohort PFOS and PFOA increased the odds of endometriosis in</td>
</tr>
<tr>
<td>Study</td>
<td>Type</td>
<td>Country</td>
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<td>Controls</td>
<td>Study Methods</td>
<td>Pollutants</td>
<td>Odds Ratio</td>
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<tr>
<td>Benzophenone</td>
<td>Cohort</td>
<td>USA</td>
<td>190 cases</td>
<td>283 controls</td>
<td>Operative cohort: Laparoscopy</td>
<td>2 OH-4MeO-BP, 2,4 OH-BP, 2,2'-OH-4MeO-BP, 2,2',4,4' OH-BP, 4 OH-BP</td>
<td>Site</td>
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<tr>
<td></td>
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<td>14 cases</td>
<td>127 controls</td>
<td>MRI (population cohort)</td>
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<td>Urine analysis</td>
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<tr>
<td></td>
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<td>14 cases</td>
<td>127 controls</td>
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<td>113 controls</td>
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<tr>
<td>Bisphenols</td>
<td>Matched</td>
<td>USA</td>
<td>190 cases</td>
<td>283 controls</td>
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<td>BPA</td>
<td>BMI, Creatinine</td>
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<tr>
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<td>cohort</td>
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<td>14 cases</td>
<td>113 controls</td>
<td>MRI (population cohort)</td>
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<td>Case</td>
<td>Italy</td>
<td>58 cases</td>
<td>11 controls</td>
<td>Laparoscopy</td>
<td>BPA and BPB</td>
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<tr>
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<td>control</td>
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<td>Mahalingaiah et al. 2014</td>
<td>Cohort</td>
<td>USA</td>
<td>Total cohort</td>
<td>Questionnaire and medical record</td>
<td>8</td>
<td>Proximity to roadways</td>
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BBP: Butyl benzyl phthalate; BEHP: Bis (2-ethylhexyl) phthalates; β-BHC: beta-benzene hexachloride; BPA: Bisphenol A; BPB: Bisphenol B; CHP: chlorinated pesticides; CX Calux assay; DEND: deep endometriosis; DL: Dioxin like; DEHP: di-2 ethylhexyl phthalate; DEP: Diethyl phthalates; DDE: dichloro-2,2-bisp-chlorophenyl ethylene; DMP: Dimethyl phthalate; DnBP: Di-n-butyl phthalates, DnOP: d-octyl phthalates; ECD: Electronic capture detector; FID: flame ionization detector; GC: Gas chromatography; LC liquid chromatography; HP: High performance; HR: high resolution; ICP-MS: Coupled plasma-mass spectrometry NDL: Non dioxin like; MCMHP: mono-2-carboxy methyl hexyl phthalate; MCHP: monocyclohexyl phthalates; MCPP: mono (3-carboxypropyl) phthalate; MECPP: mono-(2-ethyl-5-carboxypentyl) phthalate; mono-(2-ethyl-5-carboxypentyl) phthalate; MBP: mono butyl phthalate MBzP: mono-benzyl phthalates; MS: mass spectrometry; MEHP: mono-ethylhexyl phthalate, 5OH-MEHP: mono-(2-ethyl-5-hydroxyhexyl) phthalate; 5oxo-MEHP: mono (2 ethyl-5-oxohexyl) MEHP; MEP: mono-ethyl phthalate; MiBP: mono-iso-butyl phthalate; MnBP: mono-n-butyl phthalates; MMP: mono methyl phthalate; MNP: monoisononyl phthalate; MOP: monoocetyl phthalate; ON score: Ottawa New Castle scoring; TCDD: 2,3,7,8 tetrachlorodibenzop-dioxin; HCB hexachlorobenzene; HCH: hexachlorocyclohexane; Tetrachlorodiphenylyethane DDD; p,p’ DDT: dichlorodiphenyltrichloroethane; p,p’ DDE dichlorodiphenyldichloroethylene; OCP: Organochinolone pesticides; ON: Ottawa Newcastle PBB: polybrominated biphenyls; PCP: pentachlorophenole PBDE: polybrominated diphenyl ether congeners, PCB: polychlorinated biphenyls
Abbreviation list per contaminants

Perfluorochemicals:
PFC: perfluorochemicals
PFDA: perfluorodecanoic acid
PFHxS: perfluorohexane sulfonic acid
PFNA: perfluorononanoic acid
PFOA: perfluorooctanoic acid
PFOS: perfluorooctane sulfonic acid
PFDaDA: perfluorododecanoic acid
PFHpA: perfluoroheptanoic acid
PFOSA: perfluorooctanesulfonamide
PFUnDA: perfluoroundecanoic acid

Dioxins
TCDD : 2,3,7,8 tetrachlorodibenzo-p-dioxin
PCDDs: polychlorinated dibenzo-p-dioxins
1,2,3,7,8-PeCDD: 1,2,3,7,8-pentachlorodibenzo-p-dioxin
PCDFs: polychlorinated dibenzofurans
2,3,4,7,8 PeCDF: 2,3,4,7,8- polychlorinated dibenzofurans

Polychlorinated biphenyls.
PCB: polychlorinated biphenyls
DL-PCB: dioxin like polychlorinated biphenyls
NDL-PCB: non-dioxin-like PCBs
PBDE: polybrominated diphenyl

Phthalate compounds
MEHP: mono-ethylhexyl phthalate
5OH-MEHP: mono-(2-ethyl-5-hydroxyhexyl) phthalate
5oxo-MEHP: mono (2 ethyl-5-oxohexyl) MEHP
MEP: mono-ethyl phthalate
MiBP: mono-iso-butyl phthalate
MnBP: mono-n-butyl phthalates
MMP: mono methyl phthalate
MNP: monoisonyl phthalate
MOP: mono octyl phthalate

**Bisphenols**
BPA bisphenol type A
BPB: bisphenol type B
toxic equivalent factor (TEF)

**Organocholorine pesticides**
HCB hexachlorobenzene
HCH; hexachlorocyclohexane
Tetrachlorodiphenylethane DDD
p,p’ DDT: dichlorodiphenyltrichloroethane
p,p’ DDE dichlorodiphenyldichloroethylene

**Benzophenone derivates**
2OH-4MeO-BP: 2-hydroxy-4-methoxybenzophenone
2,4 OH-BP: 2,4-dihydroxybenzophenone
2,2’OH-4MeO-BP: 2,2’-dihydroxy-4-methoxybenzophenone
2,2’,4,4’OH-BP 2,2’,4,4’-tetrahydroxybenzophenone
4OH-BP: 4-hydroxybenzophenone

**Particulate matter**
PM 2.5; Particulate matter 2.5mm
PM 10: Particulate matter 10 mm
PM 25 Particulate matter 25mm
### Table: PCBs classification

<table>
<thead>
<tr>
<th>Category</th>
<th>Compounds</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Dioxin like PCBs</strong></td>
<td>77, 81, 105, 114, 118, 123, 126, 156, 157, 167, 169, 189</td>
</tr>
</tbody>
</table>
| **Anti estrogenic PCBs (Cooke et al. 2011)** | 105, 114, 126, 169 (Cooke et al. 2001) (Louis et al., 2005)  
118, 138, 163, 170, 180, 187, 194, 199 203 (Zhang et al., 2014) |
| **Estrogenic PCBs (Cooke et al. 2011)** | 18, 31, 44, 48, 52, 70, 99, 101, 126, 136, 153,188  
Cooke et al. 2001 (Louis et al., 2005)  
18, 28, 49, 52, 99, 101, 103, 110, 128 (Zhang et al., 2014) |

### Table: Analysis of cases and controls

| Laparoscopy adopted in both cases and controls | Mayani et al. 1997  
Lebel et al. 1998  
Pauwels et al. 2001  
Eskenazi et al. 2002  
De Felip et al. 2004  
Louis et. al. 2005  
Porpora et al. 2006  
Quaranta et al. 2006  
Reddy et al. 2006  
Tsuchiya et al. 2007  
Niksar et al. 2009  
Porpora et al. 2009  
Roya et al. 2009  
Simsa et al. 2010  
Martinez-Zamora et al. 2015 |
| Laparoscopy adopted only for cases | Gerhard et al. 1999  
Heiler et al. 2005  
Heiler et al. 2004  
Heiler et al. 2007 |
### Table: Definition of the cases

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<tr>
<td>Phtalates</td>
<td></td>
<td>Huang et al. 2014; Buck Louis et al. 2013; Kim et al. 2011</td>
</tr>
<tr>
<td>Organochlorine pesticides</td>
<td></td>
<td>Buck Louis et al. 2012; Porpora et al. 2009; Quaranta et al. 2006</td>
</tr>
<tr>
<td>Trace elements</td>
<td></td>
<td>Turgut et al. 2013; Pollack et al. 2013</td>
</tr>
<tr>
<td>Laparoscopy/surgery</td>
<td>Phtalates</td>
<td>Upson et al. 2013; Huang et al. 2010; Roya et al. 2008; Reddy et al. 2006; Reddy et al. 2006; Cobellis et al. 2003</td>
</tr>
<tr>
<td></td>
<td>Organochlorine pesticides</td>
<td>Upson et al. 2013; Cooney et al. 2010; Lebel et al. 1998; Gerhard and Runnebaum 1992</td>
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<tr>
<td>Trace elements</td>
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<td>Silva et al. 2013</td>
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<td>Questionnaire</td>
<td>Dioxins/PCBs</td>
<td>Fierens et al. 2003; Hoffman et al. 2007</td>
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<tr>
<td></td>
<td>Phtalates</td>
<td>Weuve et al. 2010</td>
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<tr>
<td>Partially be laparoscopy</td>
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<td>Eskenazi et al. 2002</td>
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</tbody>
</table>
References


COOKE PS, T. S., BUCHANAN DL.. *Disruption of steroid hormone signaling by PCBs.*, 2001; Lexington, KY, USA.


HASSALL, K. *The biochemistry and use of pesticides: structure, metabolism, mode of action and uses in crop protection*, 1990; London.


Chapter IV: Alternative research line

In the last year, from April to December, the PhD student realized a period of fellowship at the “University Mangiagalli” in Milan. Here she had the opportunity of comparing the obstetric outcome of spontaneous pregnancies with those one derived from medical assisted procreation, approaching this topic by both a clinical and scientific point of view. As reported in recent literature, there are substantial differences between these pregnancies and the argument becomes even more controversial if considering the new technologies, such as frozen embryo transfer and blastocyst transfer. From this scientific background, the PhD student has decided to contribute to the realization of a systematic review of literature with a meta-analysis to check the actual state of art in matter of perinatal outcomes differences after blastocyst versus cleavage state transfer.

Besides she has participated to the ideation, writing, start up and initial recruiting of a project called GENACOS (Impact of Gonadotropin GENetics Profile and OvArian Reserve on Controlled Ovarian Stimulation Outcomes). This two year study aimed at creating a predictive algorithm based on the ovarian reserve and the genetic profile of gonadotropins in order to customize the dose of gonadotropins during ovarian stimulation. It is a no profit study that is coordinated by Dr Enrico Papaleo (principal investigator) and is funded by an unrestricted grant by Merck. Its protocol has been approved by the ethical committee of coordinator and peripheral centers.

The following paragraphs are dedicated to the results of the first study and will describe GENACOS protocol.
1. Does cryopreservation influence perinatal outcomes after blastocysts versus cleavage stage transfer? A systematic review and meta-analysis

1.a Introduction

Since the first reports (Hardarson et al., 2012; Maxwell et al., 2015) extended embryo culture is becoming a widespread approach in assisted reproductive technologies. Compared with traditional cleavage-stage embryo transfer, this procedure results in a sort of “natural selection” of the most viable embryos, more similar to what usually happens during spontaneous conception in which implantation occurs only when embryos are in an advanced developmental stage (Munne et al., 2002).

Despite theoretical advantages, the clinical efficacy of blastocyst transfer versus cleavage stage transfer is debatable. In fact, while a Cochrane meta-analysis reported increased clinical pregnancy and live birth rates after blastocyst transfer (Glujovsky et al. 2016), a more recent meta-analysis did not find any statistically significant differences in these ART outcomes between blastocyst and cleavage stage transfer (Martins et al., 2016).

Data on the perinatal outcome of babies born after blastocyst transfer are even more controversial (Dar et al., 2014; Maheshwari et al., 2013). The first meta-analyses (Dar et al., 2014; Maheshwari et al., 2013) claimed that blastocyst transfer is associated with an increased risk of preterm births, very preterm births and congenital malformations. Subsequent studies did not confirm these observations (Chambers et al., 2015; Ishihara et al., 2014; De Vos et al., 2015; Oron et al., 2015).

In fact, an analysis of 43,952 singleton deliveries after transfer of blastocysts or cleavage-stage embryos did not reveal an increased risk of preterm births in blastocyst pregnancies (Chambers et al., 2015). Similarly, a recent study of 277,402 embryo transfer cycles in Japan did not find any statistically significant increase in the risk of very preterm births and preterm births after blastocyst transfer (Ishihara et al., 2014). Finally, a study of the neonatal and perinatal outcomes of 30,566 singletons did not identify any statistically significant differences in terms of congenital anomalies, or preterm and very preterm births between IVF carried out with cleavage-stage embryos and IVF carried out with blastocysts (Ginstrom Ernstad et al., 2016).

The limit of previous meta-analyses on this topic (Dar et al., 2014; Maheshwari et al., 2013; Martins et al., 2016) is that they merged data from both fresh and frozen/thawed embryo transfers without considering the effect of cryopreservation on their results.

The aim of our study was to evaluate whether cryopreservation could influence differences in perinatal outcomes between blastocyst and cleavage stage transfer. In detail, we performed a
systematic review and meta-analysis comparing perinatal outcomes after the transfer of blastocysts or cleavage-stage embryos in singleton pregnancies. We also carried out a subgroup analysis of fresh cycles alone.

1.b Methods

Protocol, eligibility criteria, information sources and search
This systematic review was conducted according PRISMA guidelines. We searched the MEDLINE (Pubmed), ISI WEB OF KNOWLEDGE and SCOPUS databases. We also searched the reference lists of relevant studies and reviews. Combinations of the following keywords and search terms were used: “blastocyst”, “cleavage stage embryo” “perinatal outcome”, “perinatal mortality”, “preterm birth” OR “premature birth”, “birth weight”, “congenital abnormalities” OR “congenital defect” OR “deformity” OR “birth defect”. No time or language restriction was adopted, and queries were limited to human studies. The reference lists of relevant reviews and articles were also hand-searched.

Study selection, data collection and data items
Three reviewers (A.C., R.B. and F.C.) evaluated titles and abstracts. Duplications were removed using Endnote online software. Disagreements were resolved by discussion between authors, and the involvement of the most experienced authors (C.A., S.G., G.D.). We included studies in which the perinatal outcomes of a singleton pregnancy born from blastocyst transfer were compared with the outcomes of cleavage stage transfer in infertile women. Case series, case report, books, congress abstracts and grey literature were not included in the analysis.

Risk of bias, summary measures and synthesis of the results
The risk of bias and quality assessment of the included studies were performed adopting the Newcastle-Ottawa Scale (NOS) (Wells et al., 2004). Three authors (A.C., R.B. and F.C.) independently assessed the risk bias for each study included. The most experienced authors (C.A., S.G and G.D.) resolved conflicts. The NOS score was used to evaluate studies included and each study was judged based on three issues: selection of the study group; comparability between groups; and ascertainment of exposed and not exposed cohorts (Wells et al., 2004). Primary outcomes were: preterm births (live birth before 37 weeks of gestation) and low birth weight (<2,500g). Secondary outcomes were: very preterm births (live birth before 32 weeks of gestation); very low birth weight (<1,500 g); small for gestational age (SGA); large for gestational age (LGA); perinatal mortality; and congenital anomalies. Data were extracted independently by three reviewers (A.C.,
R.B. and F.C.) and discrepancies were resolved by discussion with the most experienced authors (C.A., S.G. and G.D.). Publication bias of primary outcomes was assessed using funnel plots with the trim and fill method (Duval et al, 2006) and the Egger test (Egger et al., 1997).

**Statistical analysis**

Statistical analysis was carried out using Revman software (The Nordic Cochrane Centre, The Cochrane Collaboration. Review Manager version 5.3). Data were combined to a pooled odds ratio (OR). Meta-analysis was conducted using random-effect-model. Between-study heterogeneity was addressed using $I^2$ which represents the percentage of total variation in the estimated effect across studies. An $I^2$ value over 50% indicates substantial heterogeneity. $P$ values <0.05 were considered statistically significant.

1.c Results

**Study selection and characteristics**

A total of 3,697 papers was identified and 339 duplications were removed using an EndNote online library. The titles and abstracts of 3,358 papers were scrutinized and 37 full papers were assessed for eligibility. Twenty-three papers were excluded. Specifically, the data of 2 papers could not be extracted (Wang et al. 2011; Sotiroska et al., 2015); 16 papers did not fulfil our inclusion criteria. Furthermore, data replication was detected in 4 papers (Oron et al., 2015; Kallen et al., 2010; Sazonova et al., 2012). In detail, Nakashima et al: Kallen et al., Sazonova et al. extracted newborn data from the same registry used more recently by Ginstrom Ernstad et al. (2016) and Ishihara et al (2014); Oron et al. (2014) reported similar data in two papers. Moreover, the studies by Makinen et al.(2013), Wikland et al.(2010) and Kaartinen et al. (2015) were not considered because they analysed the same population of other studies included. Among papers with data replication, we chose the studies with the largest number of observations (Oron et al., 2014; Ginstrom Ernstad et al., 2016; Ishihara et al., 2014). Twelve articles were included in the qualitative/quantitative analysis (Figure 1). The characteristics of the studies included in the present study are reported in Table 1.

**Risk of bias within the study**

Risk of bias was considered “low” for studies with a Newcastle-Ottawa Scale (NOS) score above 7,“medium” for studies with a NOS score between 7 and 5, and “high” for studies a NOS score below 5 (Table 2).
Synthesis of results

Preterm birth (<37 weeks)

Preterm births were investigated in 11 studies (Maxwell et al., 2015; Chambers et al., 2015; Ishihara et al., 2014; Oron et al., 2014; Dar et al., 2013; Shchwarzler et al., 2004) (blastocyst transfer \( n = 89,709 \) vs cleavage-stage transfer \( n = 102,687 \)). The overall OR did not reveal a significant increased risk of preterm birth after blastocyst transfer than after cleavage-stage transfer (OR 1.11, 95% CI 0.99 – 1.26, \( p = 0.08, I^2 = 89\% \)). Subgroup analysis of fresh cycles (blastocyst \( n = 30,177 \) vs cleavage stage \( n = 75,021 \)) revealed a higher risk in blastocyst versus cleavage stage pregnancies (OR 1.18, 95% CI 1.06 – 1.32, \( p = 0.003, I^2 = 69\% \)) (Figure 2).

Very preterm birth (<32 weeks)

Very preterm birth was assessed in 8 studies (Kalra et al., 2012; Dar et al., 2013; Ginstrom Ernstad et al., 2016; Ishihara et al., 2010; De Vos et al., 2015; Oron et al., 2015; Maxwell et al., 2015) (blastocyst \( n = 60,488 \) vs cleavage stage \( n = 86,500 \)). Based on overall OR, there were no significant differences between blastocyst and cleavage-stage pregnancies (OR 1.05, 95% CI 0.90 – 1.23, \( p = 0.54, I^2 = 48\% \)). Subgroup analysis of fresh cycles (blastocyst \( n = 29,571 \) vs cleavage stage \( n = 74,171 \)) showed a higher risk of very preterm births after blastocyst than after cleavage-stage transfer (OR 1.16, 95% CI 1.02 – 1.32, \( p = 0.03, I^2 = 17\% \)) (Figure 3).

Small for gestational age

Small for gestation age was analysed in 7 studies (Chambers et al., 2015; Ishihara et al., 2014; Oron et al., 2014; Fernando et al., 2012; Kalra et al., 2012; Zhu et al., 2014) (blastocyst \( n = 83,123 \) vs cleavage stage \( n = 93,369 \)). Significantly fewer SGA babies was born after blastocyst than after cleavage stage transfer (OR 0.78, 95% 0.68 – 0.90, \( p = 0.0005, I^2 = 84\% \)). Subgroup analysis of fresh cycles (blastocyst \( n = 24,138 \) vs cleavage stage \( n = 65,977 \)) showed a lower risk of SGA after blastocyst than after cleavage stage pregnancy (OR 0.83, 95% CI 0.74 – 0.93, \( p = 0.001, I^2 = 35\% \)) (Figure 4).

Large for gestational age

Large for gestational age was addressed in 5 studies (Zhu et al., 2014; Fernando et al., 2012; Oron et al., 2014; Ginstrom Ernstad et al., 2016; Ishihara et al., 2014) (blastocyst \( n = 40,299 \) vs cleavage stage \( n = 45,929 \)). The overall OR showed a higher number of LGA births in blastocyst than in cleavage stage pregnancies (OR 1.23, 95% CI 1.01 – 1.50, \( p = 0.04, I^2 = 79\% \)). Subgroup analysis
of fresh cycles (blastocyst \( n = 9,382 \) vs cleavage stage \( n = 33,600 \)) did not result in a statistical significant difference between groups (OR 1.15, 95% CI 0.97 – 1.36, \( p = 0.11, I^2 = 44\% \)) (Figure 5).

**Low birth weight (<2500 kg)**

Low birth weight was investigated in 9 studies (Chambers *et al.*, 2015; Ishihara *et al.*, 2014; De Vos *et al.*, 2015; Oron *et al.*, 2014; Dar *et al.*, 2013; Kalra *et al.*, 2012; Shwarzler *et al.*, 2004) (blastocyst \( n = 86,912 \) vs cleavage stage \( n = 100,623 \)). The overall OR did not differ between blastocyst and cleavage stage pregnancies (OR 0.92, 95% CI 0.82 – 1.04, \( p = 0.20, I^2 = 86\% \)). Subgroup analysis of fresh cycles (blastocyst \( n = 27,928 \) vs cleavage stage \( n = 73,231 \)) did not reveal any statistical differences between groups (OR 1.01, 95% CI 0.92 – 1.11, \( p = 0.89, I^2 = 53\% \)) (Supplemental data 1).

**Very low birth weight (<1500 kg)**

Very low birth weight was evaluated in 6 studies (Dar *et al.*, 2013; Fernando *et al.*, 2012; Oron *et al.*, 2014; Ishihara *et al.*, 2014; De Vos *et al.*, 2015; Ginstrom Ernstad *et al.*, 2016) (blastocyst \( n = 44052 \) vs cleavage stage \( n = 53439 \)). Analysis of the overall effect size did not reveal any differences between blastocyst and cleavage stage pregnancy (OR 0.93, 95% CI 0.82 – 1.06, \( p = 0.29, I^2 = 0\% \)). Subgroup analysis of fresh cycle (blastocyst \( n = 13,135 \) vs cleavage stage \( n = 41,110 \)) revealed a comparable risk between groups (OR 0.97, 95% CI 0.81 – 1.15, \( p = 0.70, I^2 = 0\% \)) (Supplemental data 2).

**Perinatal mortality**

Perinatal mortality was assessed in 3 studies (Ginstrom Ernstad *et al.*, 2016; Dar *et al.*, 2013; Martin *et al.*, 2012) (blastocyst \( n = 8,458 \) vs cleavage stage \( n = 36,003 \)). The overall OR did not differ between blastocyst and cleavage-stage pregnancies (OR 1.43, 95% CI 0.94 – 2.17, \( p = 0.10, I^2 = 25\% \)). Subgroup analysis of fresh cycles (blastocyst \( n = 6,665 \) vs cleavage stage \( n = 30,001 \)) did not show any differences between groups (OR 1.35, 95% CI 0.95 – 1.92, \( p = 0.09, I^2 = 0\% \)) (Supplemental data 3).

**Congenital anomalies**

Congenital anomalies were addressed in 4 studies (Dar *et al.*, 2013; Martin *et al.*, 2012; Ginstrom Ernstad *et al.*, 2016; Oron *et al.*, 2015) (blastocyst \( n = 8,737 \) vs cleavage stage \( n = 36,097 \)). Analysis of the overall effect size did not reveal any differences after blastocyst vs cleavage stage
transfer (OR 0.98, 95% CI 0.84 – 1.13, \( p = 0.73 \), \( I^2 = 0\% \)). Subgroup analysis of fresh cycles (blastocyst \( n = 6,944 \) vs cleavage stage \( n = 30,095 \)) showed a comparable risk between blastocyst and cleavage stage (OR 0.99, 95% CI 0.84 – 1.17, \( p = 0.95 \), \( I^2 = 0\% \)). (Supplemental data 4).

**Risk of bias across studies**

We found no publication bias in terms of primary outcomes (Supplemental data 5): preterm birth (Egger’s test, \( p = 0.47 \)), or low birth weight (Egger’s test, \( p = 0.77 \)).
1.d Discussion

Summary of evidence

Recent systematic reviews comparing perinatal and neonatal outcomes in singleton pregnancies from cleavage stage embryos and blastocysts raised relevant concerns about the safety of extended embryo culture in IVF (Maheshwari et al., 2013; Martins et al., 2016; Dar et al., 2014). According to the latest meta-analysis, blastocyst pregnancies are associated with an increased risk of preterm births, very preterm births and LGA deliveries, and a reduced risk of SGA was observed in blastocyst pregnancies (Martins et al., 2016). Comparing with previous meta-analysis (Martins et al., 2016), we used odds ratio instead of risk ratio given the retrospective design of studies included. Furthermore, we merged fresh and frozen cycles data from Ginnstrom Ernstad et al. (2016) and Ishihara et al. (2014) as reported by Chambers et al. (2015) and Fernando et al. (2012). Finally, we provide for the first time information regarding perinatal outcome differences between blastocyst vs cleavage stage embryo transfer in fresh cycles.

Our results regarding fresh cycles are consistent with Martins et al. (2016) in terms of preterm birth, very preterm birth and SGA. However, when we included frozen/thawed cycles in our analysis, there were no differences in terms of preterm and very preterm births between blastocyst and cleavage stage pregnancies (Figures 1 and 2). Our results are in line with Chambers et al. (2015) who observed a higher odds of preterm birth in fresh cycle (OR 1.02 95% CI 0.94 – 1.11) compared with frozen cycles (OR 0.98 95% CI 0.94 – 1.11). A clear trend toward a higher risk of preterm births in fresh compared with frozen/thawed was confirmed by Ginstrom Ernstad et al. (2016). In fact, both fresh blastocyst or cleavage stage cycles showed a higher risk of preterm birth than frozen blastocyst and cleavage stage cycles respectively (7.8% fresh blastocyst vs 6.5% frozen blastocyst p = 0.09; 7.2% fresh cleavage stage vs 5.2% in frozen cleavage stage, p < 0.05).

Conversely, LGA risk appeared significantly different between blastocyst and cleavage stage embryo in fresh plus frozen/thawed cycles but not in only fresh cycle (Figure 6). Even this observation is supported by previous studies, which demonstrated a higher incidence of LGA in frozen/thawed cycle than fresh cycle (Pinborg et al., 2014).

SGA risk between groups seems not to be not influenced by frozen embryo transfer with significant reduction in cleavage stage comparing with blastocyst transfer in both fresh and frozen/thawed cycles. Regarding the effect of frozen/thawed cycles on SGA, data in literature are contradictory, with some authors who observed a reduced incidence after frozen embryo transfer comparing with fresh transfer (Kato et al., 2012) and others who detected negligible differences (Sazonova et al., 2012).
With respect of perinatal mortality we did not observed a significant risk in blastocyst pregnancy versus cleavage stage in both fresh and fresh plus frozen/thawed cycle population. Similarly, the risk of low birth weight, very low birth weight and congenital anomalies seemed to be not influenced by embryo cryopreservation procedure.

Our finding concerning congenital malformations appears in contrast with previous reports of a higher risk of this condition after blastocyst transfer (Kallen et al., 2010; Dar et al., 2014). This discrepancy may reflect the improvements made in IVF techniques and embryo culture media (Ginstrom Ernstad et al., 2016). Indeed, data coming from the same register throughout time showed that the higher risk of congenital malformations after blastocyst transfer reported by Kallen et al. (2010) disappeared in a subsequent study conducted by Ginnstrom Ernstad et al. (2016).

The safety profile of extended embryo culture is a matter of importance in clinical practice given the increasing use of this strategy in IVF centres of all world. Although, the risk of preterm birth and very preterm birth could be a cause of concern, the transfer of embryos at blastocyst stage has relevant advantages: higher implantation rate, the opportunity to select the most viable embryos avoiding transfer failure; better synchronization between endometrium and embryos at the time of transfer (Practice committees, Fertil Steril, 2013). Furthermore, extending embryo culture could favour a single embryo transfer policy, which is recommended to ensure live births for infertile couples and to reduce the risk of multiple pregnancies (ASRM, Fertil Steril, 2012). Furthermore, the fact that frozen/thawed cycles could some mitigate the adverse effect on perinatal outcomes could encourage cryopreservation in common clinical practice. This strategy offers the advantage of reducing ovarian hyperstimulation syndrome and gives the opportunity to limit the number of controlled ovarian stimulation.

Limitations
A limitation of this study is the low quality of data available. In fact, only retrospective studies are available. Furthermore, although an effect of cryopreservation is appreciable in our study, only specific meta-analysis comparing fresh with frozen cycles could definitely confirm if our observation are in the right direction. In addition, the definition of SGA and LGA deliveries is not consistent, and depend largely on the type of growth chart (Chambers et al., 2015; Ishihara et al., 2014), ethnic and racial characteristics and on the cut off values adopted. Finally, we were unable to determine whether the type of culture affected our results.
1.e Conclusion

In fresh cycles, the risk of preterm and very preterm births was significantly higher after blastocyst transfers comparing with cleavage stage embryo transfer. However, both risks are comparable between groups when frozen/thawed cycles are included. Conversely, while no differences were observed in fresh cycles, LGA births were more frequent after blastocyst transfer than after cleavage stage transfer in fresh plus frozen/thawed cycle. SGA deliveries were significantly lower after blastocyst than cleavage stage transfer both in fresh and in fresh plus frozen/thawed cycles. No differences in terms of low birth weight, very low birth weight, perinatal mortality and congenital anomalies were observed between blastocyst and cleavage stage pregnancy in both fresh and frozen/thawed cycles. Caution should be exercised in interpreting these findings given the low level of evidence available and wide heterogeneity of studies.
References


KALRA SK, RATCLIFFE SJ, BARNHART KT, COUTIFARIS C. Extended embryo culture and an increased risk of preterm delivery. *Obstet Gynecol* 2012; 120: 69-75.


Figure 1. Flow chart of papers identified, screened and reviewed

- Records identified through database searching ($n = 3,697$)
- Additional records identified through other sources ($n = 0$)

Records after duplicates removed ($n = 339$)

Records screened ($n = 1358$)

- Full-text articles assessed for eligibility ($n = 37$)
- Full-text articles excluded with reasons ($n = 321$)

Studies included in qualitative/quantitative synthesis ($n = 12$)
Fig. 2 Forrest-plot for preterm birth.

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<thead>
<tr>
<th>Study or Subgroup</th>
<th>Blastocyst Events</th>
<th>Clavtage stage Events</th>
<th>Weight</th>
<th>Odds Ratio M-H, Random 95% CI</th>
<th>Odds Ratio M-H, Random 95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chambers et al. 2015</td>
<td>1594</td>
<td>1361</td>
<td>143</td>
<td>15337</td>
<td>1.36%</td>
</tr>
<tr>
<td>Dar et al. 2013</td>
<td>648</td>
<td>3199</td>
<td>1335</td>
<td>9442</td>
<td>1.28%</td>
</tr>
<tr>
<td>De Vos et al. 2015</td>
<td>79</td>
<td>864</td>
<td>134</td>
<td>1234</td>
<td>1.30%</td>
</tr>
<tr>
<td>Fernando et al. 2012</td>
<td>165</td>
<td>1716</td>
<td>229</td>
<td>2406</td>
<td>1.13%</td>
</tr>
<tr>
<td>G. Ernsdor et al. 2016</td>
<td>351</td>
<td>4819</td>
<td>1787</td>
<td>25747</td>
<td>1.26%</td>
</tr>
<tr>
<td>Ishihara et al. 2014</td>
<td>2059</td>
<td>1339</td>
<td>671</td>
<td>14769</td>
<td>1.33%</td>
</tr>
<tr>
<td>Kaia et al. 2012</td>
<td>3157</td>
<td>14743</td>
<td>3359</td>
<td>32351</td>
<td>1.37%</td>
</tr>
<tr>
<td>Martin et al. 2012</td>
<td>42</td>
<td>433</td>
<td>64</td>
<td>750</td>
<td>1.15%</td>
</tr>
<tr>
<td>Maenw et al. 2015</td>
<td>160</td>
<td>1494</td>
<td>32</td>
<td>377</td>
<td>1.30%</td>
</tr>
<tr>
<td>Onan et al. 2014</td>
<td>43</td>
<td>279</td>
<td>12</td>
<td>94</td>
<td>1.11%</td>
</tr>
<tr>
<td>Schwabke et al. 2004</td>
<td>19</td>
<td>173</td>
<td>10</td>
<td>100</td>
<td>1.11%</td>
</tr>
</tbody>
</table>

Total (95% CI) 19799 102887 100.0% 1.11 [0.99, 1.26]

Total events 1223 11243

Heterogeneity Tau² = 0.13, Chi² = 59.41, df = 10 (P < 0.0001); I² = 69%
Test for overall effect Z = 1.72 (P = 0.08)

Only Fresh cycle

<table>
<thead>
<tr>
<th>Study or Subgroup</th>
<th>Blastocyst Events</th>
<th>Clavtage stage Events</th>
<th>Weight</th>
<th>Odds Ratio M-H, Random 95% CI</th>
<th>Odds Ratio M-H, Random 95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dar et al. 2013</td>
<td>543</td>
<td>3114</td>
<td>1331</td>
<td>9442</td>
<td>1.26 [1.13, 1.40]</td>
</tr>
<tr>
<td>De Vos et al. 2015</td>
<td>73</td>
<td>814</td>
<td>131</td>
<td>1234</td>
<td>0.63 [0.62, 1.11]</td>
</tr>
<tr>
<td>G. Ernsdor et al. 2016</td>
<td>235</td>
<td>3016</td>
<td>1421</td>
<td>18745</td>
<td>1.68 [0.64, 1.25]</td>
</tr>
<tr>
<td>Ishihara et al. 2014</td>
<td>401</td>
<td>5881</td>
<td>681</td>
<td>10928</td>
<td>1.12 [0.99, 1.28]</td>
</tr>
<tr>
<td>Kaia et al. 2012</td>
<td>3157</td>
<td>14743</td>
<td>3359</td>
<td>32351</td>
<td>1.37 [1.31, 1.44]</td>
</tr>
<tr>
<td>Martin et al. 2012</td>
<td>42</td>
<td>413</td>
<td>64</td>
<td>750</td>
<td>1.15 [0.77, 1.73]</td>
</tr>
<tr>
<td>Maenw et al. 2015</td>
<td>160</td>
<td>1494</td>
<td>32</td>
<td>377</td>
<td>1.30 [0.98, 1.84]</td>
</tr>
<tr>
<td>Onan et al. 2014</td>
<td>43</td>
<td>279</td>
<td>12</td>
<td>94</td>
<td>1.46 [0.74, 2.87]</td>
</tr>
<tr>
<td>Schwabke et al. 2004</td>
<td>19</td>
<td>173</td>
<td>10</td>
<td>100</td>
<td>1.11 [0.48, 2.49]</td>
</tr>
</tbody>
</table>

Total (95% CI) 30177 75021 100.0% 1.18 [1.06, 1.32]

Total events 4591 9033

Heterogeneity Tau² = 0.01, Chi² = 25.96, df = 8 (P = 0.001); I² = 49%
Test for overall effect Z = 2.94 (P = 0.003)
Forrest-plot for very preterm birth.

### Fresh and Frozen cycles

<table>
<thead>
<tr>
<th>Study or Subgroup</th>
<th>Blastocyst Events</th>
<th>Cleavage stage Total Events</th>
<th>Odds Ratio M-H, Random, 95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dar et al. 2013</td>
<td>95</td>
<td>251</td>
<td>1.12 [0.88, 1.43]</td>
</tr>
<tr>
<td>DeVos et al. 2015</td>
<td>17</td>
<td>1334</td>
<td>1.44 [0.73, 2.83]</td>
</tr>
<tr>
<td>Fernando et al. 2012</td>
<td>37</td>
<td>1716</td>
<td>0.92 [0.41, 2.09]</td>
</tr>
<tr>
<td>G. Ernstad et al. 2016</td>
<td>54</td>
<td>4819</td>
<td>0.90 [0.47, 1.79]</td>
</tr>
<tr>
<td>Ishihara et al. 2014</td>
<td>230</td>
<td>83300</td>
<td>1.06 [0.76, 1.49]</td>
</tr>
<tr>
<td>Kaira et al. 2012</td>
<td>414</td>
<td>14473</td>
<td>1.26 [1.13, 1.43]</td>
</tr>
<tr>
<td>Maxwell et al. 2015</td>
<td>12</td>
<td>1414</td>
<td>0.95 [0.49, 1.90]</td>
</tr>
<tr>
<td>Orn et al. 2014</td>
<td>5</td>
<td>270</td>
<td>0.55 [0.34, 0.91]</td>
</tr>
</tbody>
</table>

Total (95% CI) 60448 86500 100.0% 1.05 [0.90, 1.23]

Total events 672 1480

Heterogeneity: Tau² = 0.12; Chi² = 13.38, df = 7 (P = 0.06); P = 41%
Test for overall effect Z = 0.11 (P = 0.95)

### Only Fresh cycles

<table>
<thead>
<tr>
<th>Study or Subgroup</th>
<th>Blastocyst Events</th>
<th>Cleavage stage Total Events</th>
<th>Odds Ratio M-H, Random, 95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dar et al. 2013</td>
<td>95</td>
<td>251</td>
<td>1.12 [0.88, 1.43]</td>
</tr>
<tr>
<td>DeVos et al. 2015</td>
<td>17</td>
<td>1334</td>
<td>1.44 [0.73, 2.83]</td>
</tr>
<tr>
<td>G. Ernstad et al. 2016</td>
<td>41</td>
<td>3036</td>
<td>1.07 [0.77, 1.49]</td>
</tr>
<tr>
<td>Ishihara et al. 2014</td>
<td>42</td>
<td>5981</td>
<td>0.98 [0.48, 1.98]</td>
</tr>
<tr>
<td>Kaira et al. 2012</td>
<td>414</td>
<td>14743</td>
<td>1.28 [1.13, 1.44]</td>
</tr>
<tr>
<td>Maxwell et al. 2015</td>
<td>12</td>
<td>1482</td>
<td>0.50 [0.19, 1.34]</td>
</tr>
<tr>
<td>Orn et al. 2014</td>
<td>5</td>
<td>279</td>
<td>0.55 [0.34, 0.93]</td>
</tr>
</tbody>
</table>

Total (95% CI) 29571 74171 100.0% 1.16 [1.02, 1.33]

Total events 626 1319

Heterogeneity: Tau² = 0.31; Chi² = 7.22, df = 6 (P = 0.30); P = 7%
Test for overall effect Z = 2.18 (P = 0.03)
### Forrest-plot for SGA

#### Fresh and Frozen cycles

<table>
<thead>
<tr>
<th>Study or Subgroup</th>
<th>Blastocyst Events</th>
<th>Cleavage stage Events</th>
<th>Total Weight</th>
<th>Odds Ratio</th>
<th>M-H Random, 95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chambers et al. 2011</td>
<td>2501</td>
<td>15083</td>
<td>21.6%</td>
<td>0.91 [0.85, 0.97]</td>
<td></td>
</tr>
<tr>
<td>Fernando et al. 2012</td>
<td>141</td>
<td>2466</td>
<td>11.8%</td>
<td>0.95 [0.76, 1.19]</td>
<td></td>
</tr>
<tr>
<td>G. Erna et al. 2016</td>
<td>90</td>
<td>25747</td>
<td>14.9%</td>
<td>0.62 [0.51, 0.76]</td>
<td></td>
</tr>
<tr>
<td>Ishihara et al. 2014</td>
<td>1398</td>
<td>14759</td>
<td>21.9%</td>
<td>0.88 [0.62, 0.74]</td>
<td></td>
</tr>
<tr>
<td>Kairi et al. 2012</td>
<td>826</td>
<td>3277</td>
<td>22.0%</td>
<td>0.00 [0.74, 0.97]</td>
<td></td>
</tr>
<tr>
<td>Oron et al. 2014</td>
<td>22</td>
<td>34</td>
<td>3.0%</td>
<td>1.36 [0.41, 4.20]</td>
<td></td>
</tr>
<tr>
<td>Zhu et al. 2014</td>
<td>4</td>
<td>253</td>
<td>1.8%</td>
<td>0.44 [0.14, 1.22]</td>
<td></td>
</tr>
</tbody>
</table>

Total (95% CI): 8123 / 93359

Total events: 4982 / 6836

Heterogeneity: $T^2 = 0.02; \chi^2 = 36.75; df = 6 (P < 0.001); I^2 = 84%

Test for overall effect: $Z = 3.45 (P = 0.0015)$

#### Only Fresh cycles

<table>
<thead>
<tr>
<th>Study or Subgroup</th>
<th>Blastocyst Events</th>
<th>Cleavage stage Events</th>
<th>Total Weight</th>
<th>Odds Ratio</th>
<th>M-H Random, 95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>G. Erna et al. 2016</td>
<td>79</td>
<td>13945</td>
<td>15.9%</td>
<td>0.76 [0.69, 0.84]</td>
<td></td>
</tr>
<tr>
<td>Ishihara et al. 2014</td>
<td>357</td>
<td>10228</td>
<td>33.9%</td>
<td>0.82 [0.61, 1.05]</td>
<td></td>
</tr>
<tr>
<td>Kairi et al. 2012</td>
<td>826</td>
<td>3277</td>
<td>47.7%</td>
<td>0.10 [0.74, 0.83]</td>
<td></td>
</tr>
<tr>
<td>Oron et al. 2014</td>
<td>22</td>
<td>124</td>
<td>0.9%</td>
<td>0.16 [0.41, 3.20]</td>
<td></td>
</tr>
<tr>
<td>Zhu et al. 2014</td>
<td>4</td>
<td>253</td>
<td>1.0%</td>
<td>0.44 [0.14, 1.22]</td>
<td></td>
</tr>
</tbody>
</table>

Total (95% CI): 21419 / 65677

Total events: 1284 / 3836

Heterogeneity: $T^2 = 0.10; \chi^2 = 6.13; df = 4 (P = 0.19); I^2 = 16%

Test for overall effect: $Z = 3.24 (P = 0.001)$
Fig. 5 Forrest-plot for LGA.

### Fresh and Frozen cycles

<table>
<thead>
<tr>
<th>Study or Subgroup</th>
<th>Events</th>
<th>Total</th>
<th>Events</th>
<th>Total</th>
<th>Weight</th>
<th>M-H, Random, 95% CI</th>
<th>Odds Ratio</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fernando et al. 2012</td>
<td>104</td>
<td>1716</td>
<td>268</td>
<td>2496</td>
<td>21.9%</td>
<td>0.19 [0.81, 1.31]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>G. Ernstad et al. 2014</td>
<td>233</td>
<td>4813</td>
<td>1020</td>
<td>25747</td>
<td>20.2%</td>
<td>1.23 [1.06, 1.42]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ishihara et al. 2014</td>
<td>5730</td>
<td>33689</td>
<td>1950</td>
<td>14789</td>
<td>31.5%</td>
<td>1.45 [1.37, 1.53]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Orno et al. 2014</td>
<td>16</td>
<td>273</td>
<td>9</td>
<td>94</td>
<td>4.8%</td>
<td>0.65 [0.28, 1.50]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Zhu et al. 2014</td>
<td>17</td>
<td>98</td>
<td>311</td>
<td>2833</td>
<td>9.8%</td>
<td>1.75 [1.02, 2.99]</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Total (95% CI)           | 41299  | 45929 | 100.0% |       | 1.23 [1.01, 1.50]   |            |        |

Total events             | 8182   | 3458  |        |       |                    |            |        |

Heterogeneity Tau² = 0.03; Chi² = 13.34, df = 4 (P = 0.007); I² = 73%
Test for overall effect Z = 2.08 (P = 0.04)

### Only Fresh cycles

<table>
<thead>
<tr>
<th>Study or Subgroup</th>
<th>Events</th>
<th>Total</th>
<th>Events</th>
<th>Total</th>
<th>Weight</th>
<th>M-H, Random, 95% CI</th>
<th>Odds Ratio</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>G. Ernstad et al. 2016</td>
<td>126</td>
<td>3026</td>
<td>695</td>
<td>19745</td>
<td>34.9%</td>
<td>1.21 [1.00, 1.47]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ishihara et al. 2014</td>
<td>741</td>
<td>6581</td>
<td>1263</td>
<td>10838</td>
<td>62.6%</td>
<td>1.08 [0.98, 1.19]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Orno et al. 2014</td>
<td>18</td>
<td>279</td>
<td>9</td>
<td>94</td>
<td>3.9%</td>
<td>0.65 [0.28, 1.50]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Zhu et al. 2014</td>
<td>17</td>
<td>96</td>
<td>311</td>
<td>2833</td>
<td>8.6%</td>
<td>1.75 [1.02, 2.99]</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Total (95% CI)           | 9382   | 33680 | 100.0% |       | 1.15 [0.97, 1.36]   |            |        |

Total events             | 904    | 2278  |        |       |                    |            |        |

Heterogeneity Tau² = 0.01; Chi² = 5.33, df = 3 (P = 0.15); I² = 44%
Test for overall effect Z = 1.81 (P = 0.11)
### For the Forrest-plot for low birth weight

#### Fresh and Frozen Cycles

<table>
<thead>
<tr>
<th>Study or Subgroup</th>
<th>Blastocyst</th>
<th>Cleavage stage</th>
<th>Odds Ratio (M-H, Random, 95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cheniers et al. 2015</td>
<td>1807</td>
<td>23067</td>
<td>0.14 [0.07, 1.02]</td>
</tr>
<tr>
<td>Dai et al. 2013</td>
<td>274</td>
<td>2965</td>
<td>0.35 [0.06, 1.20]</td>
</tr>
<tr>
<td>De Vos et al. 2015</td>
<td>47</td>
<td>864</td>
<td>0.31 [0.06, 1.37]</td>
</tr>
<tr>
<td>Fernando et al. 2012</td>
<td>127</td>
<td>1716</td>
<td>1.12 [0.60, 1.98]</td>
</tr>
<tr>
<td>G. Ernst et al. 2016</td>
<td>192</td>
<td>4813</td>
<td>0.76 [0.37, 1.49]</td>
</tr>
<tr>
<td>Ishihara et al. 2014</td>
<td>3226</td>
<td>33368</td>
<td>0.32 [0.17, 0.62]</td>
</tr>
<tr>
<td>Kaira et al. 2012</td>
<td>1498</td>
<td>14623</td>
<td>0.52 [0.16, 1.26]</td>
</tr>
<tr>
<td>Oron et al. 2014</td>
<td>24</td>
<td>273</td>
<td>0.02 [0.00, 1.00]</td>
</tr>
<tr>
<td>Schwartz et al. 2004</td>
<td>17</td>
<td>173</td>
<td>0.10 [0.03, 0.38]</td>
</tr>
<tr>
<td><strong>Total (95% CI)</strong></td>
<td>86912</td>
<td>100623</td>
<td>0.62 [0.32, 1.20]</td>
</tr>
</tbody>
</table>

Total events: 72112

Heterogeneity: $I^2 = 55.33$, $Q = 1127 [P < 0.0001]$, $P = 0.00$

Test for overall effect: $Z = 1.27 (P = 0.20)$

#### Only Fresh Cycles

<table>
<thead>
<tr>
<th>Study or Subgroup</th>
<th>Blastocyst</th>
<th>Cleavage stage</th>
<th>Odds Ratio (M-H, Random, 95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dai et al. 2013</td>
<td>274</td>
<td>2965</td>
<td>0.33 [0.06, 1.80]</td>
</tr>
<tr>
<td>De Vos et al. 2015</td>
<td>47</td>
<td>864</td>
<td>0.31 [0.06, 1.37]</td>
</tr>
<tr>
<td>G. Ernst et al. 2016</td>
<td>146</td>
<td>3025</td>
<td>0.10 [0.04, 1.00]</td>
</tr>
<tr>
<td>Ishihara et al. 2014</td>
<td>756</td>
<td>5981</td>
<td>1.16 [0.95, 1.43]</td>
</tr>
<tr>
<td>Kaira et al. 2012</td>
<td>1498</td>
<td>14623</td>
<td>1.13 [0.06, 1.98]</td>
</tr>
<tr>
<td>Oron et al. 2014</td>
<td>24</td>
<td>273</td>
<td>0.09 [0.02, 0.43]</td>
</tr>
<tr>
<td>Schwartz et al. 2004</td>
<td>17</td>
<td>173</td>
<td>0.10 [0.03, 0.38]</td>
</tr>
<tr>
<td><strong>Total (95% CI)</strong></td>
<td>27920</td>
<td>73231</td>
<td>1.61 [0.92, 1.11]</td>
</tr>
</tbody>
</table>

Total events: 27644

Heterogeneity: $I^2 = 12.88$, $Q = 6 [P = 0.14]$, $P = 0.53$

Test for overall effect: $Z = 0.14 (P = 0.89)$
Supplemental data 2: Forrest-plot for very low birth weight.

### Fresh and frozen cycles

<table>
<thead>
<tr>
<th>Study or Subgroup</th>
<th>Events</th>
<th>Total</th>
<th>Events</th>
<th>Total</th>
<th>Weight</th>
<th>M-H, Random, 95% CI</th>
<th>Odds Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dar et al. 2013</td>
<td>51</td>
<td>2915</td>
<td>155</td>
<td>9109</td>
<td>16.4%</td>
<td>0.96 [0.71, 1.30]</td>
<td></td>
</tr>
<tr>
<td>DeVos et al. 2015</td>
<td>11</td>
<td>814</td>
<td>19</td>
<td>1234</td>
<td>3.0%</td>
<td>0.82 [0.39, 1.74]</td>
<td></td>
</tr>
<tr>
<td>Fernundo et al. 2012</td>
<td>31</td>
<td>1716</td>
<td>47</td>
<td>2406</td>
<td>8.1%</td>
<td>0.95 [0.60, 1.51]</td>
<td></td>
</tr>
<tr>
<td>G. Ernstad et al. 2016</td>
<td>31</td>
<td>4819</td>
<td>274</td>
<td>2574</td>
<td>14.6%</td>
<td>0.74 [0.53, 1.04]</td>
<td></td>
</tr>
<tr>
<td>Ishihara et al. 2014</td>
<td>423</td>
<td>33199</td>
<td>193</td>
<td>14786</td>
<td>57.1%</td>
<td>0.98 [0.63, 1.51]</td>
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</tr>
<tr>
<td>Orrin et al. 2014</td>
<td>5</td>
<td>279</td>
<td>3</td>
<td>94</td>
<td>0.8%</td>
<td>0.66 [0.13, 3.38]</td>
<td></td>
</tr>
</tbody>
</table>

Total (95% CI) 44052 53439 100.0% 0.93 [0.62, 1.37]

Total events 551 808

Heterogeneity: Tru²= 0.00; Chisq= 2.85, df= 5 (P = 0.72); P = 0.0%

Test for overall effect Z = 1.05 (P = 0.30)

### Only fresh cycles

<table>
<thead>
<tr>
<th>Study or Subgroup</th>
<th>Events</th>
<th>Total</th>
<th>Events</th>
<th>Total</th>
<th>Weight</th>
<th>M-H, Random, 95% CI</th>
<th>Odds Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dar et al. 2013</td>
<td>51</td>
<td>2915</td>
<td>155</td>
<td>9109</td>
<td>16.4%</td>
<td>0.96 [0.71, 1.30]</td>
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<tr>
<td>DeVos et al. 2015</td>
<td>11</td>
<td>814</td>
<td>19</td>
<td>1234</td>
<td>3.0%</td>
<td>0.82 [0.39, 1.74]</td>
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</tr>
<tr>
<td>G. Ernstad et al. 2016</td>
<td>31</td>
<td>3016</td>
<td>222</td>
<td>19746</td>
<td>71.3%</td>
<td>0.91 [0.62, 1.33]</td>
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<tr>
<td>Ishihara et al. 2014</td>
<td>85</td>
<td>5911</td>
<td>152</td>
<td>10926</td>
<td>42.5%</td>
<td>1.02 [0.78, 1.34]</td>
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</tr>
<tr>
<td>Orrin et al. 2014</td>
<td>5</td>
<td>279</td>
<td>3</td>
<td>94</td>
<td>1.4%</td>
<td>0.66 [0.13, 2.38]</td>
<td></td>
</tr>
</tbody>
</table>

Total (95% CI) 13115 41110 100.0% 0.97 [0.61, 1.53]

Total events 182 551

Heterogeneity: Tru²= 0.00; Chisq= 1.02, df= 4 (P = 0.91); P = 0%

Test for overall effect Z = 0.30 (P = 0.77)
### Supplemental data 3: Forrest-plot for perinatal mortality

**Fresh and frozen cycles**

<table>
<thead>
<tr>
<th>Study or Subgroup</th>
<th>Blastocyst Events</th>
<th>Blastocyst Total</th>
<th>Cleavage stage Events</th>
<th>Cleavage stage Total</th>
<th>Odds Ratio (M-H, Random, 95% CI)</th>
<th>Odds Ratio (M-H, Random, 95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dar et al. 2013</td>
<td>13</td>
<td>3206</td>
<td>39</td>
<td>9506</td>
<td>0.99 (0.53, 1.85)</td>
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<tr>
<td>G. Ernst et al. 2016</td>
<td>41</td>
<td>1419</td>
<td>146</td>
<td>25747</td>
<td>1.74 (1.25, 2.41)</td>
<td></td>
</tr>
<tr>
<td>Martin et al. 2012</td>
<td>1</td>
<td>433</td>
<td>2</td>
<td>750</td>
<td>0.97 (0.69, 1.38)</td>
<td></td>
</tr>
<tr>
<td><strong>Total (95% CI)</strong></td>
<td><strong>61</strong></td>
<td><strong>3603</strong></td>
<td><strong>100.0%</strong></td>
<td><strong>3603</strong></td>
<td><strong>1.43 (0.94, 2.17)</strong></td>
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<tr>
<td><strong>Total events</strong></td>
<td>61</td>
<td>189</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td><strong>Heterogeneity</strong></td>
<td><strong>Tukey = 0.04</strong>, <strong>Chi² = 2.63, df = 2 (P = 0.12), P = 25%</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td><strong>Test for overall effect</strong></td>
<td><strong>Z = 1.67, (P = 0.10)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tbody>
</table>

**Only Fresh cycles**

<table>
<thead>
<tr>
<th>Study or Subgroup</th>
<th>Blastocyst Events</th>
<th>Blastocyst Total</th>
<th>Cleavage stage Events</th>
<th>Cleavage stage Total</th>
<th>Odds Ratio (M-H, Random, 95% CI)</th>
<th>Odds Ratio (M-H, Random, 95% CI)</th>
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</thead>
<tbody>
<tr>
<td>Dar et al. 2013</td>
<td>13</td>
<td>3206</td>
<td>39</td>
<td>9506</td>
<td>0.99 (0.53, 1.85)</td>
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</tr>
<tr>
<td>G. Ernst et al. 2016</td>
<td>21</td>
<td>3026</td>
<td>107</td>
<td>19745</td>
<td>1.59 (1.03, 2.45)</td>
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<tr>
<td>Martin et al. 2012</td>
<td>1</td>
<td>433</td>
<td>2</td>
<td>750</td>
<td>0.97 (0.69, 1.38)</td>
<td></td>
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<tr>
<td><strong>Total (95% CI)</strong></td>
<td><strong>6665</strong></td>
<td><strong>30001</strong></td>
<td><strong>100.0%</strong></td>
<td><strong>30001</strong></td>
<td><strong>1.35 (0.95, 1.92)</strong></td>
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</tr>
<tr>
<td><strong>Total events</strong></td>
<td>61</td>
<td>146</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Heterogeneity</strong></td>
<td><strong>Tukey = 0.00</strong>, <strong>Chi² = 1.64, df = 2 (P = 0.14), P = 0%</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Test for overall effect</strong></td>
<td><strong>Z = 1.69, (P = 0.04)</strong></td>
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</tbody>
</table>
Supplemental data 4: Forrest-plot for congenital anomalies.

### Fresh and Frozen cycles

<table>
<thead>
<tr>
<th>Study or Subgroup</th>
<th>Blastocyst</th>
<th>Cleave stage</th>
<th>Odds Ratio</th>
<th>Odds Ratio</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Events</td>
<td>Total</td>
<td>Events</td>
<td>Total</td>
</tr>
<tr>
<td>Dar et al. 2013</td>
<td>71</td>
<td>3203</td>
<td>215</td>
<td>8906</td>
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<td>G. Ernstad et al. 2016</td>
<td>152</td>
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<tr>
<td>Martin et al. 2012</td>
<td>3</td>
<td>431</td>
<td>6</td>
<td>750</td>
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<td>Orrie et al. 2014</td>
<td>1</td>
<td>273</td>
<td>0</td>
<td>94</td>
</tr>
</tbody>
</table>

Total (95% CI) 6737 36097 100.0% 0.80 [0.84, 1.13]

Total events 231 1095

Heterogeneity Tau² = 0.00, Chi² = 0.66, df = 3 (p = 0.81); I² = 0%

Test for overall effect Z = 0.34 (p = 0.73)

### Only Fresh cycles

<table>
<thead>
<tr>
<th>Study or Subgroup</th>
<th>Blastocyst</th>
<th>Cleave stage</th>
<th>Odds Ratio</th>
<th>Odds Ratio</th>
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<tr>
<td></td>
<td>Events</td>
<td>Total</td>
<td>Events</td>
<td>Total</td>
</tr>
<tr>
<td>Dar et al. 2013</td>
<td>78</td>
<td>1206</td>
<td>215</td>
<td>8906</td>
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<td>G. Ernstad et al. 2016</td>
<td>96</td>
<td>1025</td>
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<td>Martin et al. 2012</td>
<td>3</td>
<td>433</td>
<td>6</td>
<td>750</td>
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<tr>
<td>Orrie et al. 2014</td>
<td>2</td>
<td>279</td>
<td>0</td>
<td>94</td>
</tr>
</tbody>
</table>

Total (95% CI) 6944 30095 100.0% 0.99 [0.84, 1.17]

Total events 179 385

Heterogeneity Tau² = 0.10, Chi² = 0.76, df = 3 (p = 0.80); I² = 0%

Test for overall effect Z = 0.07 (p = 0.96)
Supplemental data 5: Funnel plot and trim and firm analysis.
## 2. SYNOPSIS GENACOS

<table>
<thead>
<tr>
<th>Clinical Trial Protocol Number</th>
<th>GENACOS</th>
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<tbody>
<tr>
<td>Short Title</td>
<td>Impact of Gonadotropin GENetics Profile and OvArian Reserve on Controlled Ovarian Stimulation Outcomes</td>
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<tr>
<td>Full Title</td>
<td>A multicentre, longitudinal, cohort, interventional (venipuncture), non-pharmacological study to evaluate the impact of gonadotropin genetics together with ovarian reserve on the clinical outcome of IVF in infertile normo-gonadotropic women treated according to clinical practice</td>
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<tr>
<td>Trial Phase</td>
<td>Investigator Sponsored Interventional non-pharmacological Trial</td>
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<td>EudraCT Number</td>
<td>ENACOS</td>
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<tr>
<td>Coordinating Investigator &amp; Sponsor</td>
<td>Enrico Papaleo</td>
</tr>
<tr>
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<td>IRCCS San Raffaele Hospital, Milan, Italy</td>
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<tr>
<td>Scientific Responsible</td>
<td>Carlo Alviggi</td>
</tr>
<tr>
<td></td>
<td>“University “Federico II”, Naples, Italy</td>
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<tr>
<td>Clinical centers</td>
<td>• Centro Scienze della Natalità, Ob-Gyn Dept., San Raffaele Hospital, Milan, Italy.</td>
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<td>• Dipartimento di Neuroscienze, Scienze Riproduttive ed Odontostomatologiche”, University “Federico II, Naples, Italy</td>
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<tr>
<td></td>
<td>• ANDROS Day Surgery Clinic, Reproductive Medicine Unit, Palermo, Italy</td>
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<tr>
<td>Centralized Laboratory</td>
<td>Clinica Zucchi - Biogenesi</td>
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<tr>
<td></td>
<td>Prof. Manuela Simoni</td>
</tr>
<tr>
<td></td>
<td>Unit of Endocrinology, Department of Biomedical, Metabolic and Neural Sciences, University of Modena and Reggio Emilia, Italy</td>
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<td>Azienda USL of Modena, Italy</td>
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<th>Dr. Daniel Santi</th>
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<table>
<thead>
<tr>
<th>Scientific advisor</th>
<th>Prof. Kypros Nicolaides</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>King’s College – London, UK</td>
</tr>
</tbody>
</table>

| Equipment required      | E8 expert HD live with sonoAVC, v-SRI, crossXbeam, HDflow, VCI with omiview + |
|                        | High resolution 4D transvaginal probe Referent for ultrasound procedure standardization and data managment: |

| Planned trial period    | 2 years of data collection |
| (first subject in-last subject out) |
2a. Objectives

The main objective of this study is to develop a predictive algorithm, based on Patients Ovarian Reserve (estimated through AFC = antral follicle count) and gonadotropin genetic profile, to define the personalized gonadotropin dose for controlled ovarian stimulation during Assisted Reproductive Techniques.

Infertile female patients with normal ovarian reserve (OR) will be treated according to clinical practice and the difference between expected (through AFC) and retrieved oocytes will be assessed in relation to gonadotropin genetic profile.

2b. Scientific Background

The number of oocytes collected remains the key prognostic marker in women undergoing Assisted Reproduction Technologies (ART), and growing evidence shows that an optimal – rather than a maximal – oocyte yield is the preferred achievement after controlled ovarian stimulation (COS) when fresh embryo transfer is scheduled. In fact, live birth rates steadily increase when six to fifteen oocytes are retrieved, while further hyper-response is associated with lower implantation rates, increased obstetrical risks and increased risk of OHSS in the fresh cycle (Verberg et al., 2009; Sunkara et al., 2011; 2015).

Criteria to select the proper starting dose of FSH have not yet been identified (Farquhar et al., 2014). If no previous cycles have been performed, the choice will be based on criteria as women's age and markers of ovarian reserve. On these bases different algorithms have been proposed, having demographic, anthropometrics and ovarian reserve indicators as independent variables and the number of retrieved oocytes as endpoint (Howles et al., 2006; La Marca et al., 2012). Despite promising results, these algorithms have been developed in retrospective analyses of limited series and need to be validated in adequately sized prospective studies.

Although the “concept” of ovarian reserve dramatically changed the approach to COS, both hormonal and functional biomarkers do not allow a perfect personalization of therapies in IVF cycles for every patient, as it has been demonstrated that some patients can have a hyper-response when stimulated with very low quantity of drugs and hypo-response in case of stimulation with high dosages of gonadotropins. The explanations for this discrepancies fall in two theoretical categories. The first is related to the relevant variability of ovarian reserve tests.
In particular, AMH measurements are associated with high inter-assay variability, whereas AFC shows limits in terms of inter-operator interpretation. These potential biases could affect both development and use of algorithms. In this scenario, moving from “subjective” to “objective” is crucial. Recently, it has been proposed the use of ultrasonographic automated- 3D follicular count to improve inter-reliability in AFC. This idea moved from the evidence that manual measurement of follicles with 2D ultrasonography (US) is often inaccurate and “time consuming”. Furthermore, it is associated with a degree of measurement error as follicles can be missed or counted more than once. It has recently suggested that the Automatic Volume Calculation (AVC), a software that identifies and quantifies hypoechoic regions within a 3D dataset and provides automatic estimation of their absolute dimensions, mean diameter and volume, may represent a valid alternative to standard 2D evaluation. Several studies demonstrate that AVC significantly improves the inter-observer reliability of AFCs and also provides highly reliable measures of number and the size of antral follicles when compared with standard 2D evaluation (Scheffer et al., 2002; Raine-Fenning et al., 2007; Jayaprakasan et al., 2008; Raine-Fenning et al., 2008; Deb et al., 2009; 2010; Ata et al., 2011).

The second potential limit of algorithms which are only based on ovarian reserve is that they did not include genetic characteristics of patients. Lessons from last decade clearly indicate that women with similar demographic, anthropometric and gonadotropin profiles may have dramatically different ovarian reserve. Nevertheless, there is also evidence that, even in presence of similar ovarian reserve, the “sensitivity” of follicles to exogenous FSH can differ. For instance, two women having the same AFC may display diverse Follicular Output RaTe (FORT [Genro et al., 2011]), meaning a different percentage of follicles reaching maturity with a given FSH dose. Follicular sensitivity to exogenous FSH may be related to a a “poly-genic trait”. This hypothesis is supported by studies correlating ovarian response to gonadotropins with the analysis of polymorphisms (genetic variants whose frequency is higher than 1%) of gonadotropins and their receptors. The majority of the studies were focused on the single nucleotide polymorphisms (SNPs) of FSH receptor gene (FSHR) and in particular on two substitutions of exon 10. The first SNP (rs6165) determines the nucleotide change (at position 919) of adenine with guanine and the consequent amino acid substitution of threonine with alanine, at codon position 307 (Thr307Ala). The second SNP determines the nucleotide change (at position 2039) of adenine with guanine, leading to the amino acid substitution of asparagine with serine at position 680 (Asn680Ser) (Aittomaki et al., 1995). These polymorphisms are in linkage disequilibrium. There is an increasing body of evidence suggesting that carriers of
680Ser show “hypo-sensitivity” to gonadotropin and require higher doses of exogenous FSH to achieve adequate ovarian response to COS (Behre et al., 2005).

More recently, another polymorphism in the promoter region of the FSHR, in position -29, was identified (Wunsch et al., 2005). This polymorphism is characterized by the substitution of guanine with adenine (GGAA in GAAA) and it seems to reduce the expression of the FSHR. Our preliminary experience (data on file) suggest that carriers of this polymorphism also display reduced sensitivity to standard r-hFSH doses.

The aim of this multicentre, longitudinal, cohort, interventional non-pharmacological study is to develop an algorithm for identifying the starting dose of r-hFSH by evaluating the impact of gonadotropin genetics together with ovarian reserve on the clinical outcome of IVF in infertile normo-gonadotropic women treated according to clinical practice.

This project introduces new crucial aspects with respect to previously reported models, including i) prospective design; ii) analysis of genetic aspects and iii) highly standardization of 3D automated AFC. Finally, this study will also provide information on the impact all independent variable (including genetics and ovarian reserve) on the first stages of pregnancy. More specifically, they will be correlated with the outcome of first trimester screening. In fact, data in literature showed that conception by IVF is associated with increased risk for adverse pregnancy outcome (Chaveeva et al., 2010), such as early preeclampsia (Carbone et al., 2011). The IVF procedure itself or the features of the subgroup of patients requiring IVF might affect the risk of obstetric and birth outcomes This study might lead to further understanding of the etiology and pathology of adverse obstetric outcome in these pregnancies.

2c. Methodology

Multicenter, longitudinal, prospective, interventional, non-pharmacological cohort study, enrolling women attending clinical Centers of medically assisted reproduction (ART) with a normal ovarian reserve (the cohort).

The study protocol consists in the record all the diagnostic and clinical outcome parameters, according to clinical practice, for the following patients cohort:

- Normo-gonadotropin patients (basal FSH ≤12 IU/L) with normal Ovarian Reserve (8
\( \leq AFC \leq 16 \) that will be treated according to clinical practice with the following protocol:

- 14-21 days with contraceptive
- r-hFSH treatment 150 IU/day for the whole stimulation period, starting from the 5\textsuperscript{th} day following contraceptive cessation (vs basal assessment day 2-3 of the cycle)
- Gn-RH antagonist starting from day 6 of r-hFSH stimulation;
- hCG triggering with at least two follicle >16 mm or GnRH-agonist triggering in case of risk of OHSS.
- Fresh embryo transfer. In case of OHSS risk and/or in case of progesterone rise on the day of hCG administration (\( \geq 2 \text{ ng/mL} \)), all the embryos will be cryopreserved.

Additional blood samples will be collected for gonadotropin genetic polymorphisms assessment and a serum sample for AMH, Estradiol (E\textsubscript{2}), LH and Progesterone (P\textsubscript{4}).

Planned number of subjects: All subjects attending the Centers, in the study period, who match study inclusion criteria. At least 374 patients have to be enrolled.

Primary endpoints: Number of retrieved vs. expected oocytes through AFC in relation to -29 FSHReceptor (FSHR) Single Nucleotide Polymorphism (SNP).

The AFC is measured with AVC and performed until the 5\textsuperscript{th} day in a natural cycle prior to the contraceptive cycle.

Secondary endpoints:

- N° of follicles >10 mm; >12 mm; >14 mm; >16 mm; >18 mm;
- AMH ng/mL performed in a natural cycle prior to the contraceptive cycle;
- E\textsubscript{2} level at basal, Day 5 and hCG day (centralized);
- LH IU/L, basal, day 5 and hCG day (centralized);
- Progesterone ng/mL on the hCG day (centralized);
- r-hFSH dose (IU)/oocytes;
- Duration FSH treatment;
% of mature oocytes;

Fertilization rate;

Number of embryos developed, transferred and cryopreserved;

Implantation Rate;

Pregnancy Rate per started cycle;

Pregnancy rate per embryo transfer;

Ongoing pregnancy rate per started cycle.

Endpoints I trimester:

Rate of chromosomal abnormalities;

Rate of early preeclampsia;

Rate of small for gestational age;

Association analysis with additional SNPs: FSHR680; LHR291; FSHB-211; v-beta LH.

Inclusion Criteria:

First stimulation cycle;

Second stimulation cycle with “normo-response” to previous COS;

Caucasian women;

Eumenorroids normo-gonadotrophic women;

34 ≤ Age ≤39;

basal FSH ≤12 IU/L;

Ovarian Reserve between 8≤AFC≤16;

Body Mass Index; 18≤ BMI ≤27;

IVF/ICSI indication;
SmPC drugs criteria.

Exclusion Criteria:

Ovarian cyst > 12 mm the day of the beginning of COS;

PCOS according to Rotterdam;

POR according to ESHRE Criteria;

Second stimulation cycle;

Endometriosis III-IV stage;

Intervention: Gonadotropin Genetic Profile (FSHR-29; FSHR680; LHR291; FSHB-211- v beta LH).

Reference therapy dose/mode of administration/dosing schedule: None.

Planned trial and treatment duration per subject: From initial visit until the end of assisted reproductive technique into two years of study.

Statistical methods

Several studies evaluated follicle stimulating hormone receptor (FSHR) gene in vitro activity, considering the single nucleotide polymorphisms in the promoter region of the gene (position -29) (rs134205). They found that transcriptional activity of the A allele was 56±8% of that observed for the G allele. In particular, Desai et al. found a significant reduction in relative FSHR mRNA expression for the A/A genotype in 100 women attending assisted reproductive techniques. Considering the dominant model for the FSHR gene polymorphism, they found a relative FSHR mRNA expression of 0.12 for G/G allele, 0.07 for G/A and 0.02 for A/A. Thus, power analysis was performed (by G*Power software, version 3.1.9.2) assuming a variation of 0.075 of relative FSHR mRNA expression produced by two genotypes. Difference between two independent means was considered as statistical analysis, α error probability was set at 0.05 and allocation ratio of 0.89, considering previous observational study. Considering a statistical power of 95%, the sample size is 164 women for first group and 148 women for second one. The total number of at least 374 women to be observed, considering a drop-out rate of 20%.

Kolmogorov-Smirnov test will be used for valuation of parameters’ distribution. Mann-Whitney and Kruskall-Wallis tests will be used for comparison of non-parametric variables, whereas
ANOVA will be used for parametric ones.

Main parameter of this study will be the number of retrieved oocytes and this parameter will be compared to AFC, gonadotropin doses and genetic profile. The variables predictive of the number of retrieved oocytes will be assessed by backwards stepwise multiple regression. Backward selection of parameters will be applied, using Wald $p<0.05$ for entry and $p>0.1$ for removal. The variables reaching the statistical significance in multivariate regression analysis will be used in the calculation for the model elaborated in order to find the predictive algorithm. This algorithm will be implemented evaluating the association between genotype and response, creating dominant and recessive haplotype models, according to genotypes frequency.

Statistical analysis will be performed using the ‘Statistical Package for the Social Sciences’ software for Macintosh (version 20.0; SPSS Inc., Chicago, IL). Genotypic association tests assuming codominant, dominant, recessive, overdominant or log-additive genetic models will be performed using SNPstats. Linkage disequilibrium will be evaluated using SNPStat.
References


DESAI, S. S., et al. 'Follicle-stimulating hormone receptor polymorphism (G-29A) is associated with altered level of receptor expression in Granulosa cells', J Clin Endocrinol Metab, 2011; 96 (9), 2805-12.


GENRO VK, GRYNBERG M, SCHEFFER JB, ROUX I, FRYDMAN R, FANCHIN R. Serum anti-Müllerian hormone levels are negatively related to Follicular Output RaTe (FORT) in normo-cycling women undergoing controlled ovarian hyperstimulation. Hum


