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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.

TAB 1 DIAGNOSTIC CRITERIA FOR PCOS.

NIH Consensus 1990 (all required)	Rotterdam Consensus 2003 (two out of three required)	AEPCOS definition 2006 (androgen excess and one other criterion)
Clinical and/or biochemical hyperandrogenism	Clinical and/or biochemical hyperandrogenism	Clinical and/or biochemical hyperandrogenism
Oligo/amenorrhea, anovulation	Oligo/amenorrhea, anovulation	Oligo/amenorrhea, anovulation
	Polycystic ovaries appearance on ultrasound	Polycystic ovaries appearance on ultrasound

Exclusion of other androgen excess disorders: NC-CAH, Cushing's syndrome, androgen secreting tumors, hyperprolactinemia, thyroid diseases, drug-induced androgen excess. Other causes for anovulation should also been excluded.

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.

TAB 2 PCOS PHENOTYPES.

Classic PCOS	Ovulatory PCOS	PCOS without hyperandrogenism
Hyperandrogenism and anovulation with or without PCO	Hyperandrogenism and PCO	Anovulation and PCO
More severe menstrual disturbances and hyperandrogenism	Lesser degrees of hyperandrogenism	Minor menstrual irregularity
Higher prevalence of total and abdominal obesity and metabolic syndrome	Lower prevalence of metabolic syndrome and milder forms of dyslipidemia	Metabolic profile often similar to normal women
Higher prevalence of T2DM and cardiovascular risk factors		

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.

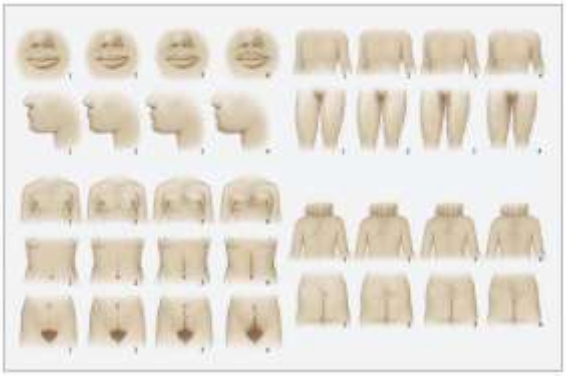
It's important to measure hormones in early follicular phase. Elevated free Testosterone (T) levels are observed in approximately 70% of PCOS patients. The vast majority of the abnormal values are in the form of free T, with the sole measurement of total T adding little to the diagnosis. The present 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 psycho logic 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[5]. 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.

2 The Ultrasonographic findings related to insulin resistance in patients with polycystic ovarian syndrome: a retrospective observational study

2.a 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 estro-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/m^2; 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 ^{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% (0.69-93.2), 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 insulinemic 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.

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

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.

Characteristics	**Type A ovary** (*n* = 33)	**Type B ovary** (*n* = 45)	***p* value**
Age (years)	28.4 ± 5.4	29 ± 5.8	0.61
BMI (kg/m^2)	23.7 ± 2.3	43.1 ± 56	0.048
WHR	0.9 ± 0.1	0.9 ± 0.1	0.0001
HOMA	1.5 ± 0.8	4.9 ± 2.8	0.0001
LH (UI/L)	9.25 ± 6.3	5.2 ± 3.3	0.002
Ovary volume(cm^3)	12.1 ± 4	14.3 ± 5.6	0.048
SHBG (nmol/l)	53.5 ± 23.5	28 ± 11.6	0.0001
Ferriman-Gallwey	11.6 ± 2.9	10.5 ± 3.4	0.14
Free testosterone (pg/ml)	2.6 ± 2.6	2.1 ± 1.7	0.27
DHEA-S (µg/dl)	224.7 ± 221.7	164.4 ± 109.2	0.14
17-OH-P (ng/ml)	1.8 ± 1.5	1.2 ± 1.3	0.057

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).

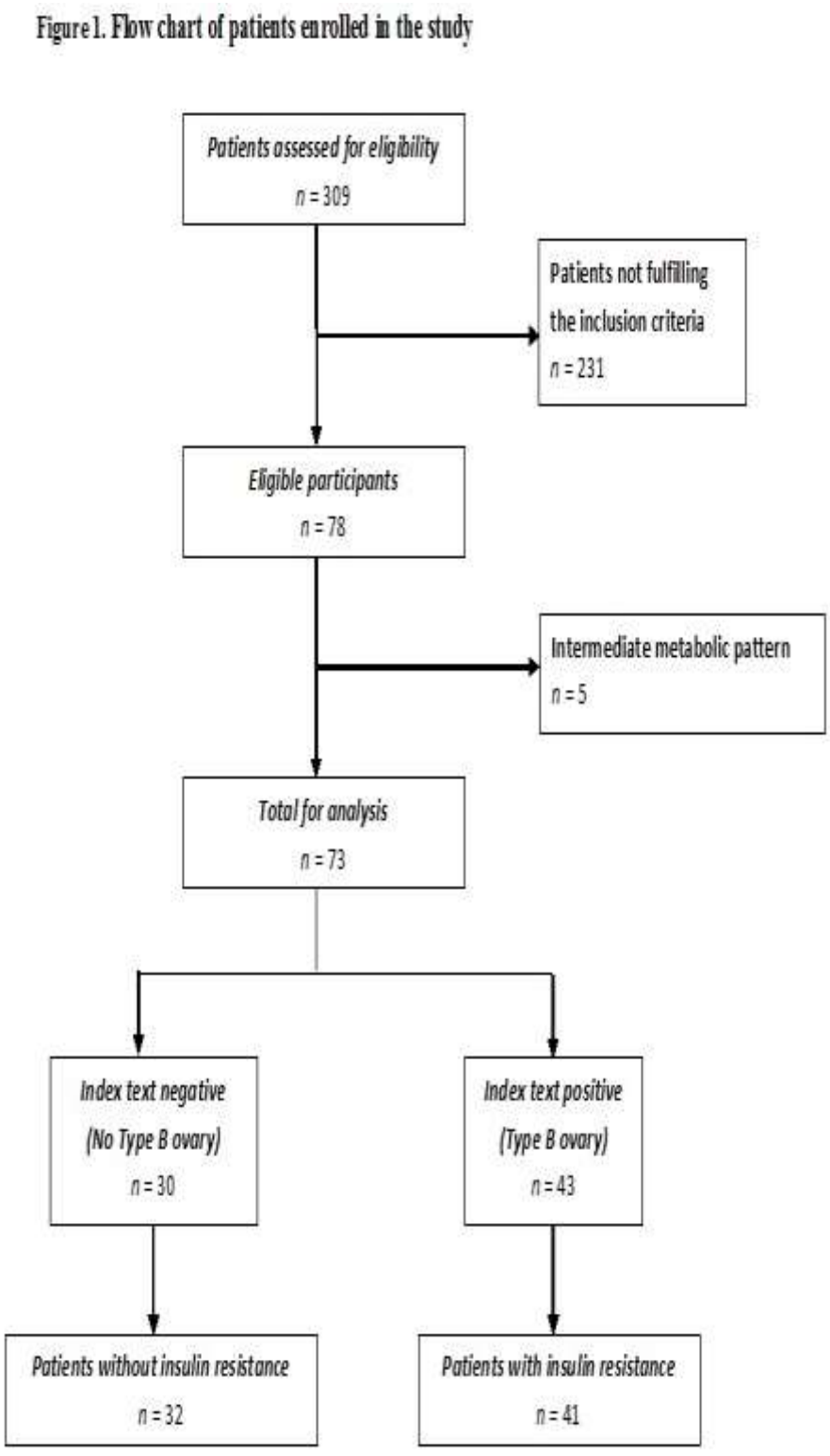


Fig.3 Example of median ovarian section with the ovarian and stromal total areas defined. Calipers are positioned so as to encircle the total gonad circumference (A1) and the stromal component circumference (A2). The stroma/total area (S/A) was also calculated.

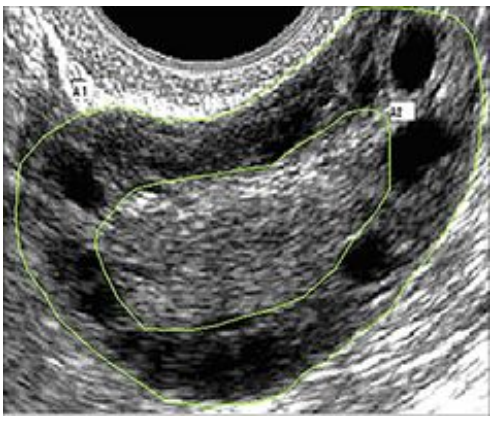


Fig.4 Sonographic pattern of a type A ovary (A). Note the typical rosary arrangement of follicles and the easily recognizable hyperechogenicity that results from thickening of theca (B). The stromal hypervascularity is clearly visible in C. Secondary aspects are the dominance of follicles with a diameter > 4 mm and the predominance of longitudinal diameter (D).

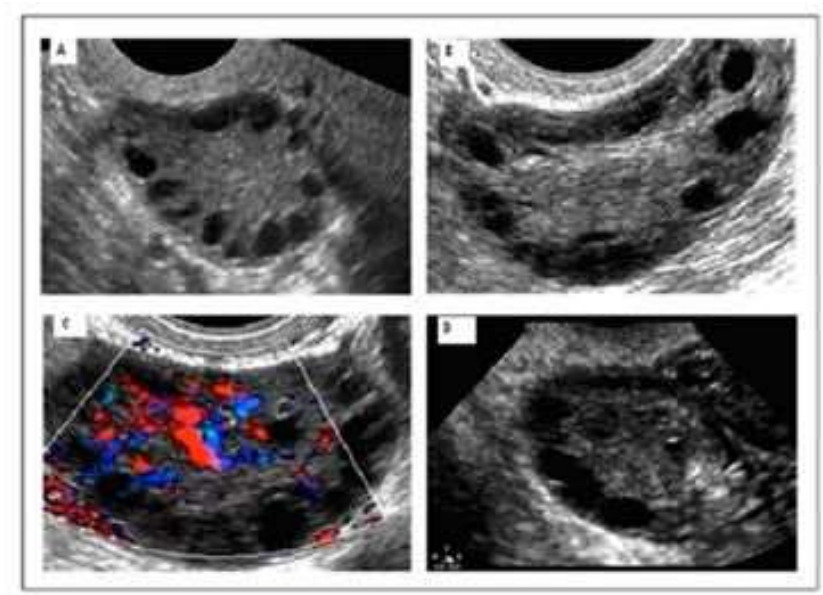
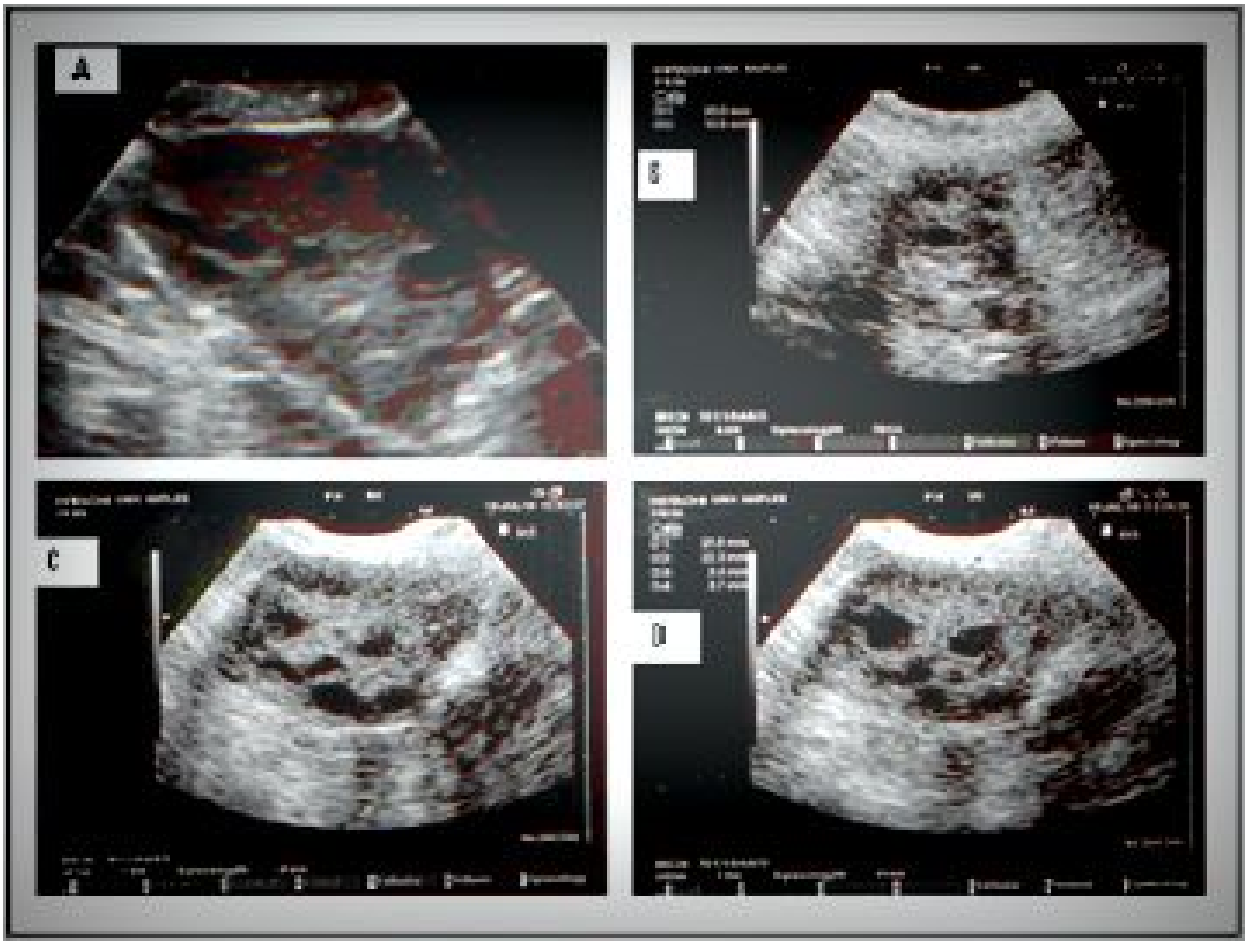


Fig.5 Sonographic pattern of a type B ovary (A). Note the ubiquitous arrangement of follicles, and the absence of central echogenicity (B). Characteristic signs of the type B ovary are a more "globular" gonad versus a type A ovary (C), with attenuation of the typical dominance of longitudinal diameter, 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 endometriosic 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 endometriosic 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.

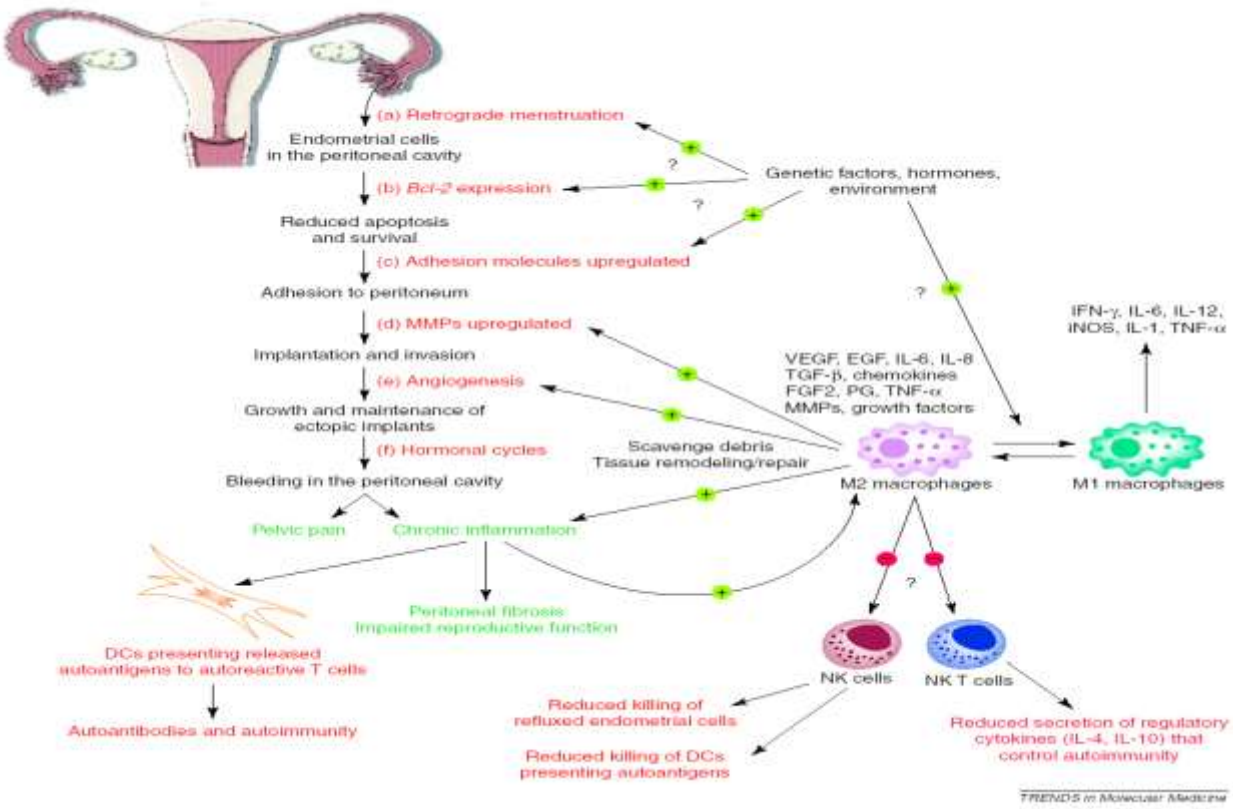
Independently of the different theories, the peritoneal environment seems to play an important role in pathogenesis. Molecular adhesion of cells and other factors produced by macrophages are responsible for the adhesion of fragments of endometrial tissue on the peritoneum. Later in the accession process, 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 endometriosic lesions.

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 Enviromental 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 endometriosic 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 endometriosic 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 congeners 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 s 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, 49, 66, 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_a 1.01 95% CI 0.83, 1.24; Population OR_a 1.06 95% CI 0.66, 1.71); PCB 138 (Operative OR_a 0.98 95% CI 0.80, 1.20 Population OR_a 0.80 95% CI 0.40, 1.63); PCB 153 Operative OR_a 0.87 95% CI 0.71, 1.07 Population OR_a 1.13 95% CI 0.68, 1.89; PCB 170 (Operative OR_a 0.79 95% CI 0.62 1.02 Population OR_a 0.72 95% CI 0.32, 1.62); PCB 180 (Operative OR_a 0.91 95% CI 0.74, 1.12 Population OR_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_a 0.72 95% CI 0.55-0.93) and PCB 156 (OR_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_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 estrogenic 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 estrogenic 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.

Phtalates 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-β-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 bisphenols 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

Perflurochemicals 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 perflurochemicals 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 Langendonckt *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 valuated 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.

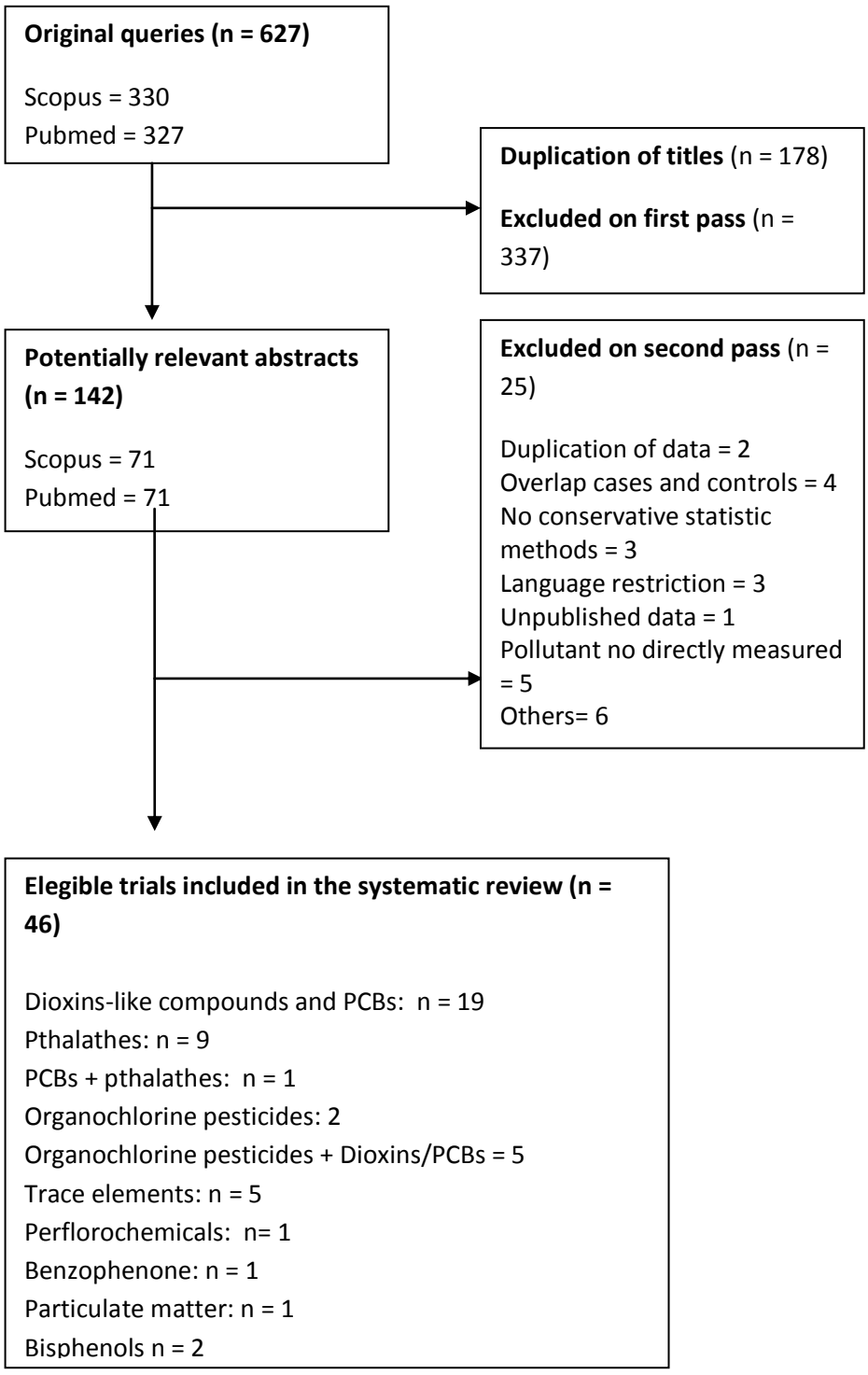
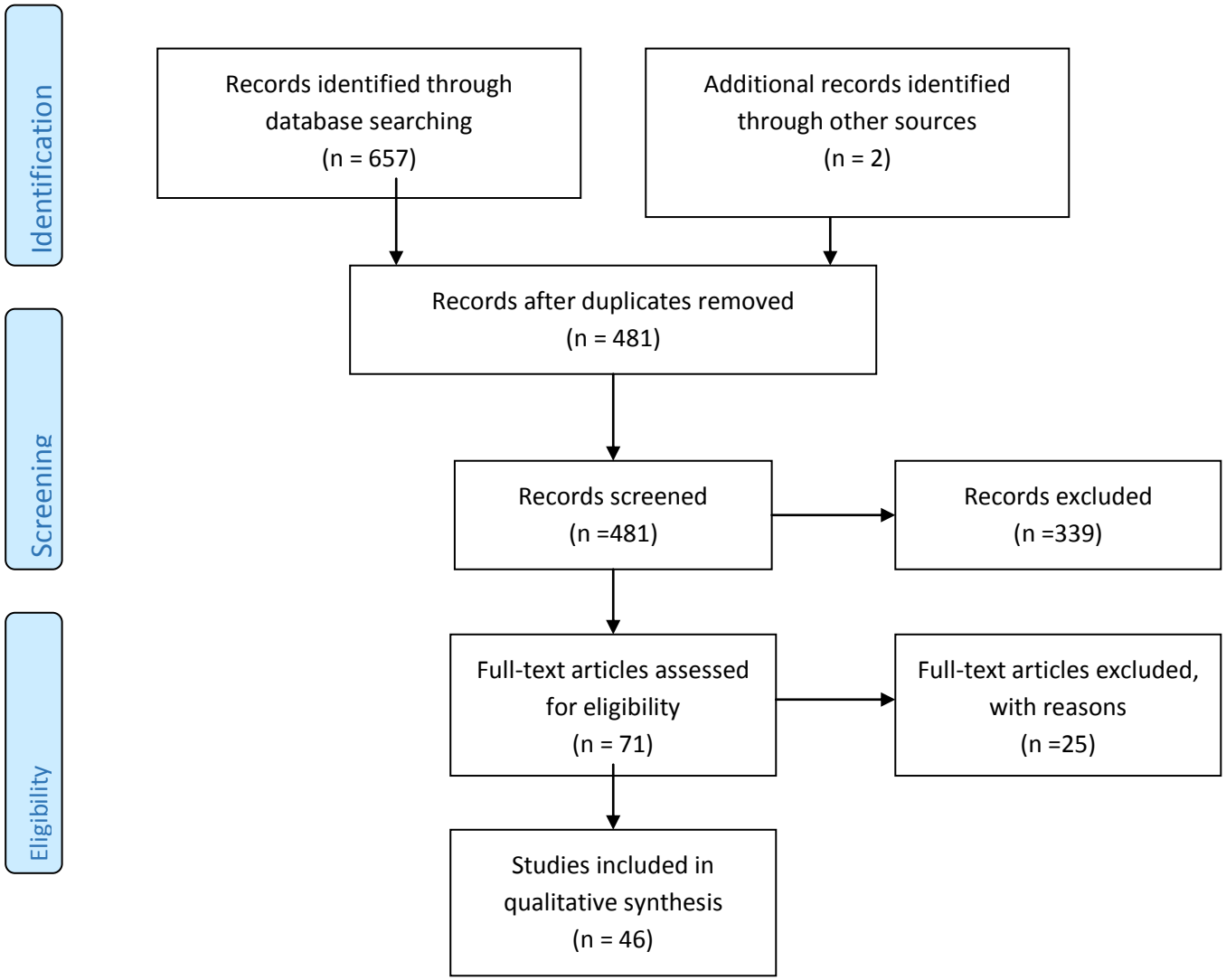


Fig. 2b Flow chart according PRISMA statement.



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.

Pollutant	**Author, Year, (ref)**	**Study design**	**Population**		**Diagnosis method**	**ON Score**	**Method (pollutant analysis)**	**Exposure**	**Exposure evaluation**	**Confounders adjusted for**	**Results: effect estimation**
			Country	**Individuals**							
Dioxins and PCB											
	Martinez-Zamora *et al.* 2015	Case control	Spain	30 cases 30 controls	Laparoscopy and histology (DEND)	7	HR-GC/HR-MS	Omentum	7 PCDDs 10 PCDFs 12 DLPCBs	Breastfeeding	2,3,4,7,8-PeCDF, 2,3,7,8-TCDD, 1,2,3,7,8-PeCDD were significantly higher in patients with DIE Dioxins and DL-PCBs (PCB-114; PCB-156; PCB-189; PCB-126) were significantly higher in patients with DIE
	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	PBDEs: 47, 99, 100, 153, 154, 209; DLPCBs: 114,118,156, 167, 189. NDLPCBs: 18, 28, 44, 49, 52, 66, 74, 87, 99, 101, 128, 138, 146, 149, 151, 153, 157,	Age BMI Breastfeeding Lipids Codipine	Crude OR significant for PCB 28,151,201 PCBE 47 with reduced ORa PCBs 74,156 with reduced ORa PCB 153 showed a significant OR when breastfeeding was removed from the model

				113 controls					170, 172, 177, 178, 180, 183, 187, 194, 195, 196, 201, 206, 209.		
	Vichi *et al.* 2012	Case control	Italy	63 cases 63 controls	Laparoscopy and histology	7	HR-GC/ Ion trap MS	Serum analysis	NDL PCB: 28, 52, 101, 138, 153, 170, 180 DL PCB 105, 118, 156, and 167.	Breastfeeding and Lipid	PCBs 118, 138, 153, 170, 180 are associated with a significant increased risk of endometriosis
	Cai *et al.* 2011	Case control	Japan	10 cases 7 controls	Laparoscopy	5	GC/HR-MS	Peritoneal fluid Serum	Dioxin compounds: 7 PCDDs; 10 PCDF and all dioxin like PCBs	Lipid	Higher levels of dioxin TEQ (PCDDs and PCDFs) in peritoneal fluid of endometriosis patients (OR: 2.5; 95% CI: 1.17–5.34; *p :* 0.035). No differences in serum
	Trabert *et al.* 2010	Case control	USA	251 cases 538 controls	Laparoscopy (cases) Interview (controls)	9	HR-GC/HR-MS	Serum analysis	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)	Breastfeeding Lipid	No statistical differences
	Sisma *et al.* 2010	Case control	Belgium	96 cases 105 controls	Laparoscopy and histology	7	CALUX	Serum analysis	Dioxin compounds		Increased risk of endometriosis (OR: 2.5; 95% CI: 1.17–5.34; *P* = 0.035)

	Roya *et al.* 2009	Case control	India	86 cases 91 controls	Laparoscopy	5	GC flame ionization detector	Plasma analysis	PCB 1, 5, 29, 98		Significant increase in endometriosis group, related with stage
	Porpora *et al.* 2009	Case control	Italy	80 cases 78 controls	Laparoscopy and histology	8	CALUX HRGC/MS ion trap	Serum analysis	DLPCBs (105,118,156,167) NDLPCBs (28,52,101,138,153,167, 170-180) Dioxin compounds;	Age, smoking habits, BMI, Weight changes	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 2.01-11.0); NDL PCB 180 (OR = 3.05 (1.25–7.42) Total PCBs (OR = 5.63; 95% CI 2.25-14.1)
	Niskar *et al.* 2009	Case control	Usa	60 case 64 controls	Laparoscopy and histology	8	HRGC/ HRMS	Serum analysis	3 PCDD; 2 PCDF; DLPCBs (118,126, 156,169); NDLPCBs (138,153,180); ppDDE;	Lipid analysis and breast feeding	No significant differences
	Tsuchiya *et al.* 2007	Case control	Japan	79 cases (31 stage I-II) (48 stage III IV) 59 controls	Laparoscopy	7	GS/HRMS	Serum analysis	8 PCDDs 10 PCDFs 4 DL PCBs 36 NDLPCBs	Lipid analysis	No significant differences
	Hoffman *et al.*. 2007	Cohort	USA	79 cases 864 controls	Questionnaire		GC/ electron capture detection	Serum analysis	PBBs PCBs	Age Household income, Menopausal status, PBB levels	Increased incidence of endometriosis among women exposed to moderate PCB and high PCB

										PCB level	
	Porpora *et al.* 2006	Case control	Italy	40 cases 40 controls	Laparoscopy	8	HRGC/MS ion trap	Serum analysis	2 DLPCB (118, 156) 6 NDL (28, 52, 101, 138, 153, 180)	metabolic disease, gravidity, parity, weight changes	Significant increase serum concentration of PCBs in patients with endometriosis
	Quaranta *et al*, 2006	Case control	Italy	10 cases 8 controls	Laparoscopy	6	HRGC/MS ion trap	Serum analysis	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
	Reddy *et al.* 2006[a]	Case control	India	85 cases 135 controls	Laparoscopy	7	GC	Plasma analysis	PCB: 1, 5, 29; 98 (no coplanar)		PCBa 1, 5, 29 98 higher in cases
	Buck Louis *et al.* 2005	Case control	USA	32 cases 52 controls	Laparoscopy	9	GC/electron capture detection	Serum analysis	7 DLPCBs (105, 114, 118, 126, 167, 169, 189) 55 NDLPC		Borderline significance for anti-estrogenic PCBs (p: 0,08) that are lower in endometriosis; no differences for estrogenic PCBs
	Heilier *et al* 2005	Case control	Belgium	25 END 25 DEND 21 controls	END:laparoscopy DEND:histology	6	GC/HR-MS	Serum analysis	17 PCDD/Fs, 12 dioxin like PCBs	standardization for BMI and age	Dioxin like PCBs were significantly higher in adenomyiosis; PCDD/PCDF were significantly higher in cases than controls; significantly increase risk of adenomyiosis and endometriosis
	Heilier *et al.* 2004	Case control	Belgium	10 DEND 7 END 10 controls	END:laparoscopy DEND:histology	6	GC/HR-MS	Serum analysis	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

	De Felip *et al*. 2004	Case control	Italy/Belgium	23 cases 17 controls	Laparoscopy and histology	6	GC-HRMS	Serum analysis	17 PCDD/PCDFs, 12 dioxin-like PCBs		No significant difference in cases and controls; lower body burden levels in Italian population
	Fierens *et al*. 2003	Case control	Belgium	10 cases 132 control	Questionnaire	8	GC.HRMS	Serum analysis	17 PCDD/Fs 11 NDLPCBs (3,8,28,52,10,138,153, 180,194,206,209), 5 DLPCBs (77, 81, 118,126)		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
	Eskenazi *et al*. 2002	Cohort	Italy	19 cases 277 controls (14 surg cases 39 surg contr).	Laparoscopy no in all cases	7	GC/ HRMS	Serum analysis	TCDD		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 >100 ppt doubled not significant risk for endometriosis among woman with TCDD >100ppt.
	Pauwels *et al*. 2001	Case control	Belgium	42 cases 27 controls	Laparoscopy	8	CALUX GC-ECD	Serum analysis	Dioxins compounds 3 NDLPCBs (138,153,180)	BMI Alcohol intake	No statistical differences
	Lebel *et al*. 1998	Case control	Canada	86 cases 70 controls	Laparoscopy	7	GC/ ECD	Plasma analysis	12 NDLPCBs (28, 52, 99, 101, 105, 128, 138, 153, 170, 180, 183, and 187) 2 DLPCBs (118, 156)	Age BMI	No statistical differences

	Mayani *et al.* 1997	Case control	Israel	44 cases 35 controls	Laparoscopy	4	GC/ MS	Blood analysis	TCDD	lipid	Non significant OR 7.6 (95% CI 0.87–169.7).
	Gerhard and Runnebaum 1992	Case control	Germany	28 cases 441 control	Laparoscopy		n.a.	Blood analysis	NDL-PCB 128,153,180		PCB (128, 153, 180) significantly higher among endometriosis group
Phthalates											
	Huang *et al.* 2014	Case control	Taiwan	44 cases (16 adeno-myosis) 69 controls	Laparoscopy and histology	7	LC/ Electro-spray ionization MS	Urinary phthalates metabolites	MMP, MEP, MnBP, MBzP, MEHP, 5oxo-MEHP, 5OH-MEHP		Endometriosis/adenomyosis women showed a marginally increased level of urinary MEHP only
	Buck Louis *et al.* 2013	Matched cohort	USA	190 cases 283 controls (operative cohort) 14 cases 113 controls (population cohort)	Laparoscopy/Surgery and histology (operative cohort) MRI (population cohort)	5	Enzymatic deconjuga-tion and solid phase extraction	Urinary phthalate metabolites	MECPP, MCMHP, 5oxo-MEHP, 5OH-MEHP, MEHP; mCPP, MMP, MEP, MiBP, MnBP, MCHP, MBzP, MNP, MOP, MBP BPA	Age BMI Creatinine	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

	Upson *et al.* 2013	Case control	USA	92 cases 195 controls	Laparoscopy/ Surgery	7	LC-MS	Urinary phthalate metabolites	MEHP, 5OH-MEHP, 5oxo-MEHP, MECPP, MBzP, MEP, MiBP, MnBP	Age Education Smoking Alcohol	Inverse associatiotn MEHP (4th vs 1st quartiles (aOR 0.3, 95% CI: 0.1–0.7)
	Kim *et al.* 2011	Case control	Korea	97 cases 169 controls	Surgical and histology	8	LC- MS	Plasma analysis	MEHP, DEHP	Pregnancy BMI	MEHP Elevated in endometriosis women OR 1.020 (CI 95% 1.003–1.038 $p = 0.20$)
	Weuve *et al.* 2010	Cross-sectional	USA	1020 controls 87 cases	Questionnaire			Phthalate metabolites	MEHP, MBP, MEP, MBzP, 5OH-MEHP, 5oxo-MEHP	Age Ethnicity Age menarche Pregnancy Breast feeding	Positive association for MBP and endometriosis or leiomymatosis and weak inverse association of MEHP and endometriosis or leiomymatosis
	Huang *et al* 2010	Case control	Taiwan	29 controls 29 endometr. 16 adeomios	Surgery and histology		LC-MS	Urine	MMP, MEP, MnBP MBzP, MEHP, 5oxo-MEHP and 5OH-MEHP		Creatinine adjusted MnBP higher in cases
	Roya *et al.* 2008	Case control	India	99 cases 135 controls	Laparoscopy	5	HP-LC	Serum analysis	DMP, DEP, DnBP BBP. BEHP		DMP, DEP, DnBP BBP. BEHP higher in study group; each compound significantly correlated with endometriosis severity

	Reddy *et al.* 2006[b]	Case control	India	49 cases 38 controls	Laparoscopy	6	HP-LC - GC	Plasma analysis	DnBP, BBP, DnOP DEHP		DnBP, BBP, DnOP DEHP higher in endometriosis groups and significantly correlated with endometriosis severity
	Reddy *et al.* 2006[a]	Case control	India	85 cases 135 controls	Laparoscopy	7	GC	Plasma analysis	DnBP; DEHP; DnOP. BBP.		Phatelates esters higher in cases Higher value of both pollutant in advanced stage.
	Cobellis *et al.* 2003	Case control	Italy	35 cases 24 controls	Laparoscopy	7	HP-LC	Serum Peritoneal fluid	DEHP MEHP		Significantly higher serum levels of DEHP in patients with endometriosis
Organochlorine pesticides											
	Upson *et al.* 2013	Case control	USA	248 cases 538 controls	Laparoscopy	7	Isotope-dilution GC-HR-MS	Serum analysis	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

									and mirex.		
	Buck Louis *et al.* 2012	Matched Cohort	USA	Operative cohort: 190 cases 283 controls Population cohort: 14 cases 113 controls	Laparoscopy/ Surgery and histology (operative cohort) Magnetic resonance (population cohort)	8	GC/HRMS electronic capture detector	Serum Omentum	OCPs: HCB, HCH, γ-HCH, β-HCH, oxychlordane, cis- and trans-nonachlor, cis- and trans-chlordane, p,p´-DDT p,p´-DDE o,,p´-DDT	Age BMI Breastfeeding Lipids Codipine	γ-HCH associated with endometriosis in operative cohort β-HCH associated with endometriosis in population cohort
	Cooney *et al.* 2010	Case control	USA	29 cases 51 controls	Laparoscopy	8	GC electron-capture	Serum analysis	Aldrin, β-BHC; DDE; HCB, mirex, trans-nonachlor	Total serum lipids Smoking Other organochlorine pesticides	HCB associated with increased odds of endometriosis
	Porpora *et al.* 2009	Case control	Italy	80 cases 78 controls	Laparoscopy and histology	8	HRGC/MS ion trap	Serum analysis	HCB; pp' DDE	Age, smoking habits, BMI, Weight changes	No statistical differences
	Quaranta *et al*, 2006	Case control	Italy	10 cases 8 controls	Laparoscopy and histology	6	HRGC/MS ion trap	Serum analysis	*p,p'*-DDE		significant increase serum concentration of p,p'-DDE in patients with endometriosis
22	Lebel *et al.* 1998	Case control	Canada	86 cases 70 controls	Laparoscopy	7	GC/ ECD	Plasma analysis	α chlordane, γ chlordane, p,p´ DDT, p,p' DDE, aldrin, β HCH, HCB, mirex, oxychlordane, *cis*-nonachlor, *trans*-nonachlor	Age BMI	No statistical differences

24	Gerhard and Runnebaum 1992	Case control	Germany	28 cases 441 control	Laparoscopy/Surgery		n.a.	Blood analysis	HCH, HCB, DDT, DDE, DDD		No association was reported
Trace elements											
	Turgut *et al.* 2013	Case control	Turkey	31 cases 41 controls	Laparoscopy/surgery and histology	5	Copper: Specto-metry Ceruloplasmin; nephelo-metric assay	Serum analysis	copper and ceruloplasmin		Cu (μg/ml): 1088.00 ± 273.58 811.20 ± 265.77 < 0.001
	Silva *et al.* 2013	Case control	Sri Lanka	50 cases 50 controls	Laparoscopy/Surgery	5	The total-reflection X-ray fluorescence Atomic absorption spectroscopy	Blood analysis	lead; nichel; cadmium		Nickel higher in endometriosis group Lead: not significant Cadmium: not significant
	Pollack *et al* 2013	Matched cohort	USA	Operative cohort: 190 cases 283 controls Population	Laparoscopy/ Surgery and histology (operative cohort) MRI (population cohort)	8	ICP-MS	Blood and urine analysis	Urine: antimony, arsenic (As), barium, beryllium, cadmium, cesium, chromium, cobalt (Co), copper, lead, manganese, mercury,	Age BMI, Smoking, Race, Vitamin use Creatinine, Parity/gravidity	In the operative cohort, blood cadmium was associated with a reduced odds of diagnosis (aOR = 0.55; 95% CI: 0.31, 0.98), while urinary chromium and copper reflected an increased odds

				cohort: 14 cases 113 controls					molybdenum, nickel (Ni), tellurium, thallium, tin, tungsten, uranium and zinc. Blood: Cadmium, lead		(aOR = 1.97; 95% CI: 1.21, 3.19
	Heilier *et al*. 2006	Case control	Belgium	119 cases 25 controls	Laparoscopy	5	electro thermal graphite furnace atomic absorption spectro-metry	Serum and urine for cadmium, serum for lead	cadmium, lead		No correlation for cadmium; lower serum levels of lead in endometriosis
	Heilier *et al*. 2004	Case control	Belgium	38 cases (25 endometriosis13 adeno-myosis) 21 controls	laparoscopy in cases, controls clinical exam	7	electrothermal graphite furnace atomic absorption spectrometry	Serum and urine analysis	cadmium	Age Smoking	No difference in serum among the groups; in urine, not difference significant, but the mean was slightly higher in adenomyosis
Perfluorochemicals											
	Buck Louis *et al*. 2012	Cohort	USA	Operative cohort 190 cases 283 controls Population	Laparoscopy and histology (operative cohort) MRI (population cohort)	5	HP-LC/MS	Serum analysis	PFDA, PFHxS, PFNA, PFOA, PFOS, PFDoDA, PFHpA, PFOSA, PFUnDA	Age BMI Parity	Serum perfluorooctanoic acid and perfluorononanoic acid associated with endometriosis in operative cohort PFOS and PFOA increased the odds of endometriosis in

				cohort 14 cases 113 controls							advanced stages in both population. Results were moderately attenuate with parity adjustment
Benzophenone											
	Kunisue *et al.* 2012	Cohort	USA	Operative cohort 190 cases 283 controls Population cohort 14 cases 127 controls	Laparoscopy (operative cohort) MRI (population cohort)	5	LC-MS	Urine analysis	2 OH-4MeO-BP, 2,4 OH-BP, 2,2'OH-4MeO-BP 2,2',4,4'OH-BP 4 OH-BP	Site Hair colour	2,4OH-BP increase the odds of an endometriosis in the operative cohort
Bisphenols											
	Buck Louis *et al.* 2013	Matched cohort	USA	190 cases 283 controls (operative cohort) 14 cases 113 controls (population cohort)	Laparoscopy (operative cohort) MRI (population cohort)	5	HP-LC+ MS		BPA	Age BMI Creatinine	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
	Cobellis *et al.* 2009	Case control	Italy	58 cases 11 controls	Laparoscopy	4	HPLC-MS	Serum analysis	BPA and BPB		BPA and BPD not detected in control group. No odds provided

Particulate matter											
	Mahalingaiah *et al.* 2014	Cohort	USA	Total cohort 84060	Questionnaire and medical record	8	Proximity to roadways Estimated outdoor levels of particulates		Air pollution PM 10; PM 2.5; PM 10-25;	Age, Calendar time Race, BMI, Smoking, Parity Oral Contraception. Menarche, Infertility Rotating shift work Region	No association observed

BBP:Butyl benzyl phthalate; BEHP: Bis (2ethylexyl) 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: Dimethyil 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: monoisonoyl phthalate; MOP: monooctyl phthalate; ON score: Ottawa New Castle scoring; TCDD: 2,3,7,8 tetrachlorodibenzo-p-dioxin; HCB hexachlorobenzene ; HCH; hexachlorocyclohexane; Tetrachlorodiphenylethane 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

PFDoDA: 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: monoisonoyl phthalate

MOP: monooctyl 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	
Dioxin like PCBs	77, 81, 105, 114, 118, 123, 126, 156, 157, 167, 169, 189
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

	Trabert *et. al* 2010
Other diagnostic method	Carpenter *et al.* 2001 (electronic database) Carpenter *et al* 2003 (electronic database) Fierens *et al.* 2003 (questionnaire)
No specified diagnostic method	Gerhard *et al.* 1992

Table: Definition of the cases		
Gold standard (Laparoscopy/surgery and histology)	Dioxins/ PCBs	De Felip *et al.* 2004; Porpora *et al.* 2006; Quaranta *et al.* 2006; Niksar *et al.* 2009; Porpora *et al*. 2009; Simsa *et al.* 2009; Buck Louis *et al*. 2012; Vichi *et al.* 2012; Martinez and Zamora *et al* 2015
	Phtalates	Huang *et al.* 2014; Buck Louis *et al.* 2013; Kim *et al.* 2011
	Organochlorine pesticides	Buck Louis *et al.* 2012; Porpora *et al.* 2009; Quaranta *et al.* 2006
	Trace elements	Turgut *et al.* 2013; Pollack *et al.* 2013
Laparoscopy/surgery	Dioxins/ PCBs	Mayani *et al.* 1997; Lebel 1998; Gerhard *et al.* 1992; Pauwels *et al.* 2001; Heiler *et al.* 2004; Heiler *et al.* 2005; Buck Louis *et al.* 2005; Reddy *et al.* 2006, Tsuchiya *et al.* 2007; Roya *et al*. 2009; Cai *et al.* 2011; Trabert *et al*. 2010
Laparoscopy/surgery	Pthalates	Upson *et al.* 2013; Huang *et al.* 2010; Roya *et al.* 2008; Reddy *et al*. 2006; Reddy *et al.* 2006; Cobellis *et al.* 2003
	Organochlorine pesticides	Upson *et al.* 2013; Cooney *et al.* 2010; Lebel *et al.* 1998; Gerhard and Runnebaum 1992
	Trace elements	Silva *et al.* 2013
Questionnarie	Dioxins/PCBs	Fierens *et al.* 2003; Hoffman *et al.* 2007
	Phthalates	Weuve *et al.* 2010
Partially be laparoscopy		Eskenazi *et al.* 2002

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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 **GEN**etics Profile and Ov**A**rian Reserve on **C**ontrolled **O**varian **S**timulation 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*., Sazanova *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.

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

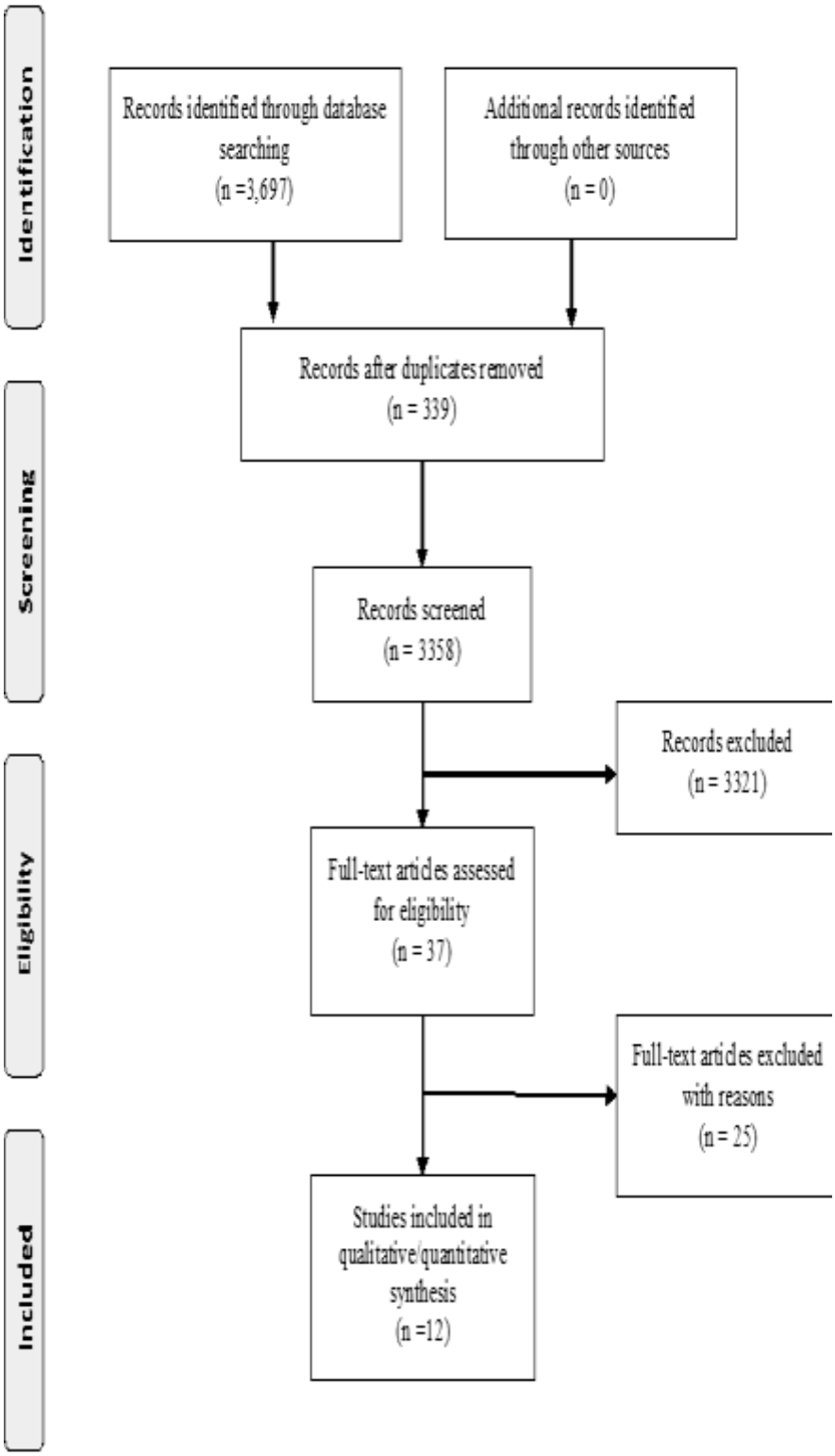


Fig. 2 Forrest-plot for preterm birth.

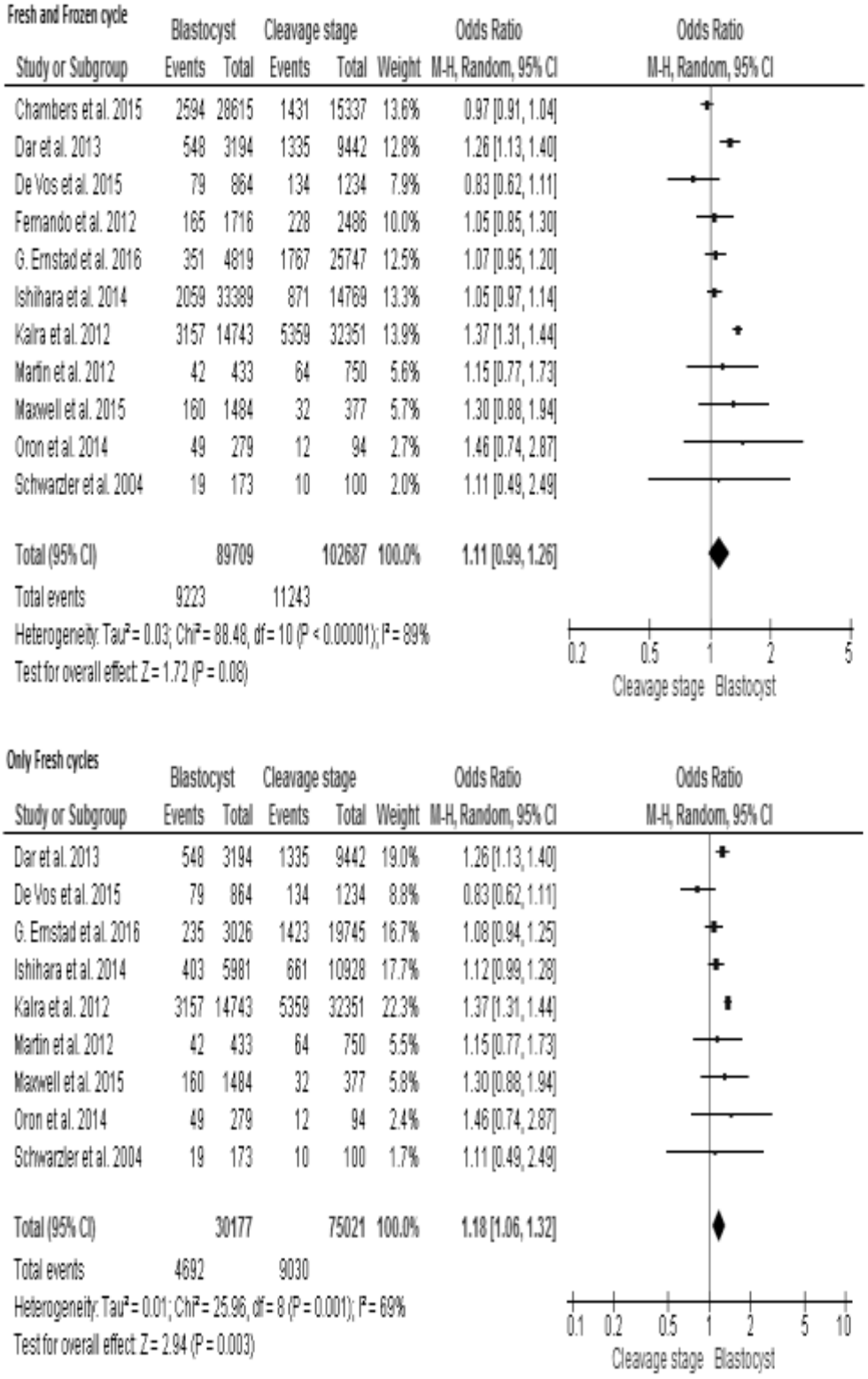
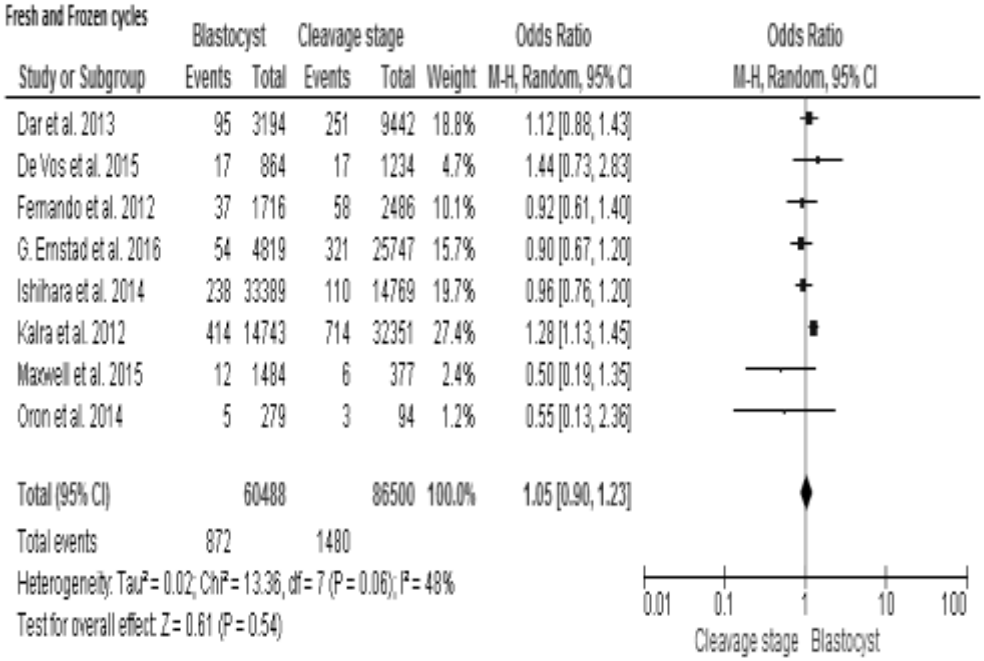


Fig. 3 Forrest-plot for very preterm birth.



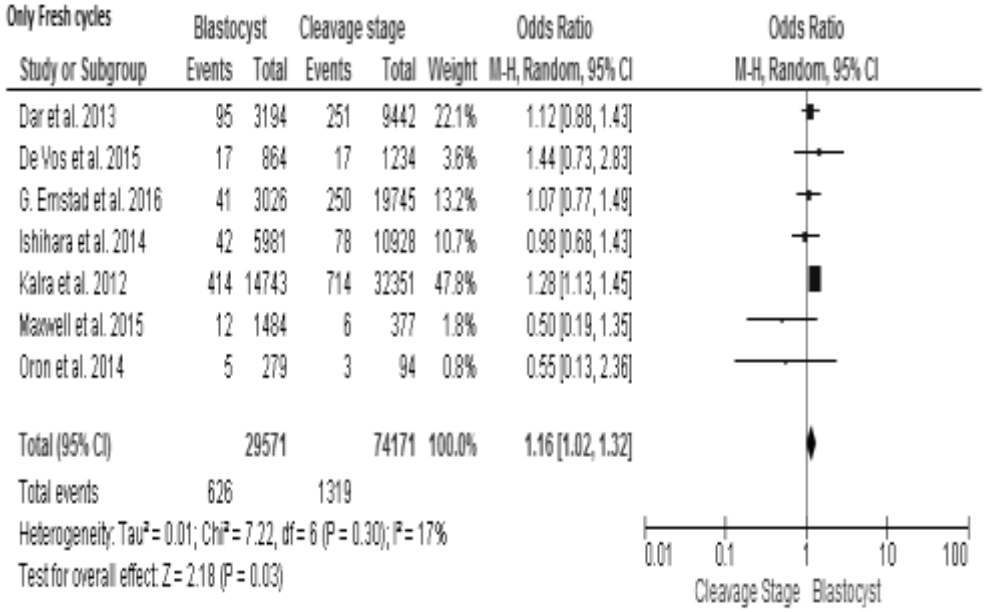


Fig. 4 Forrest-plot for SGA.

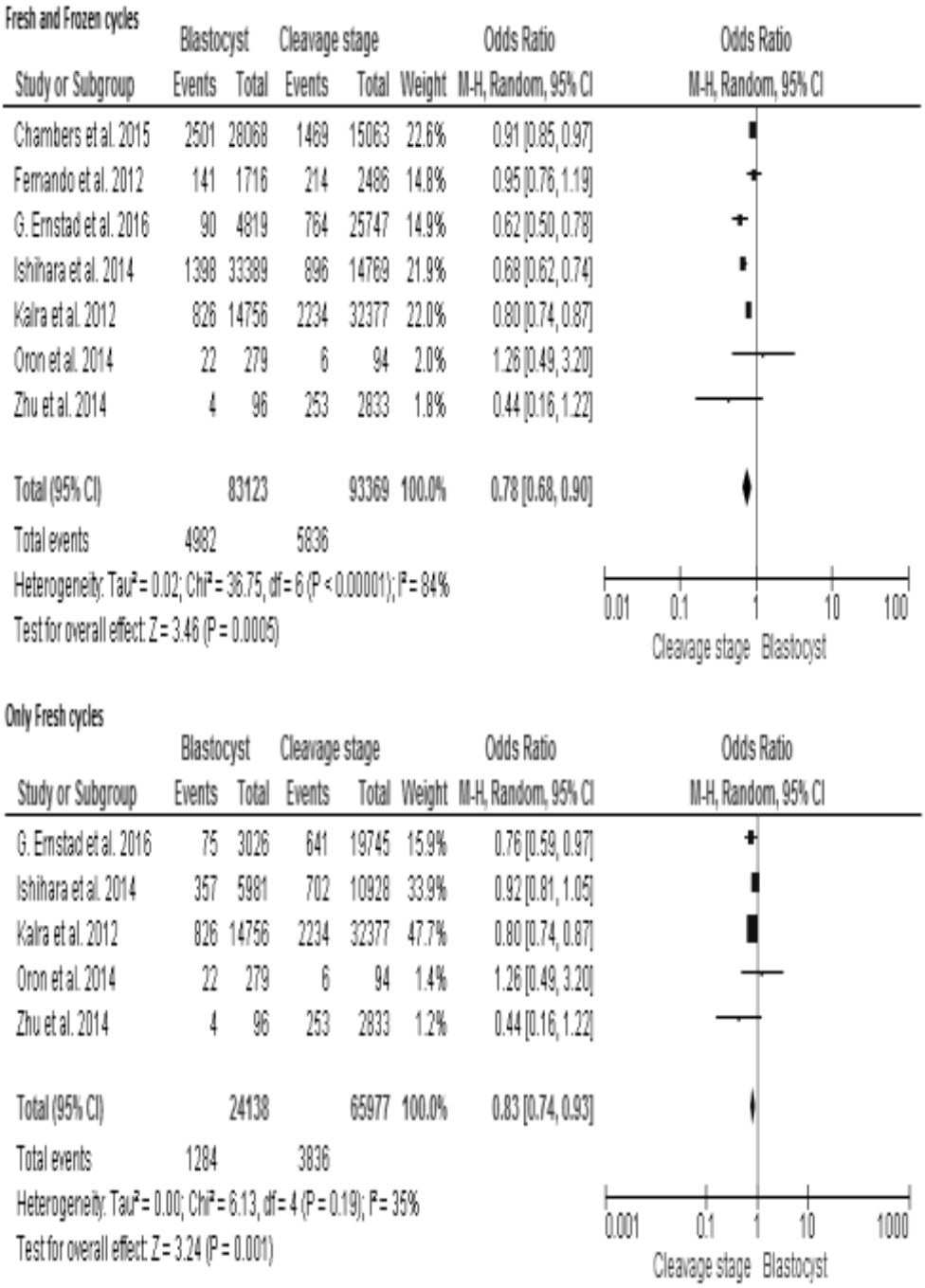
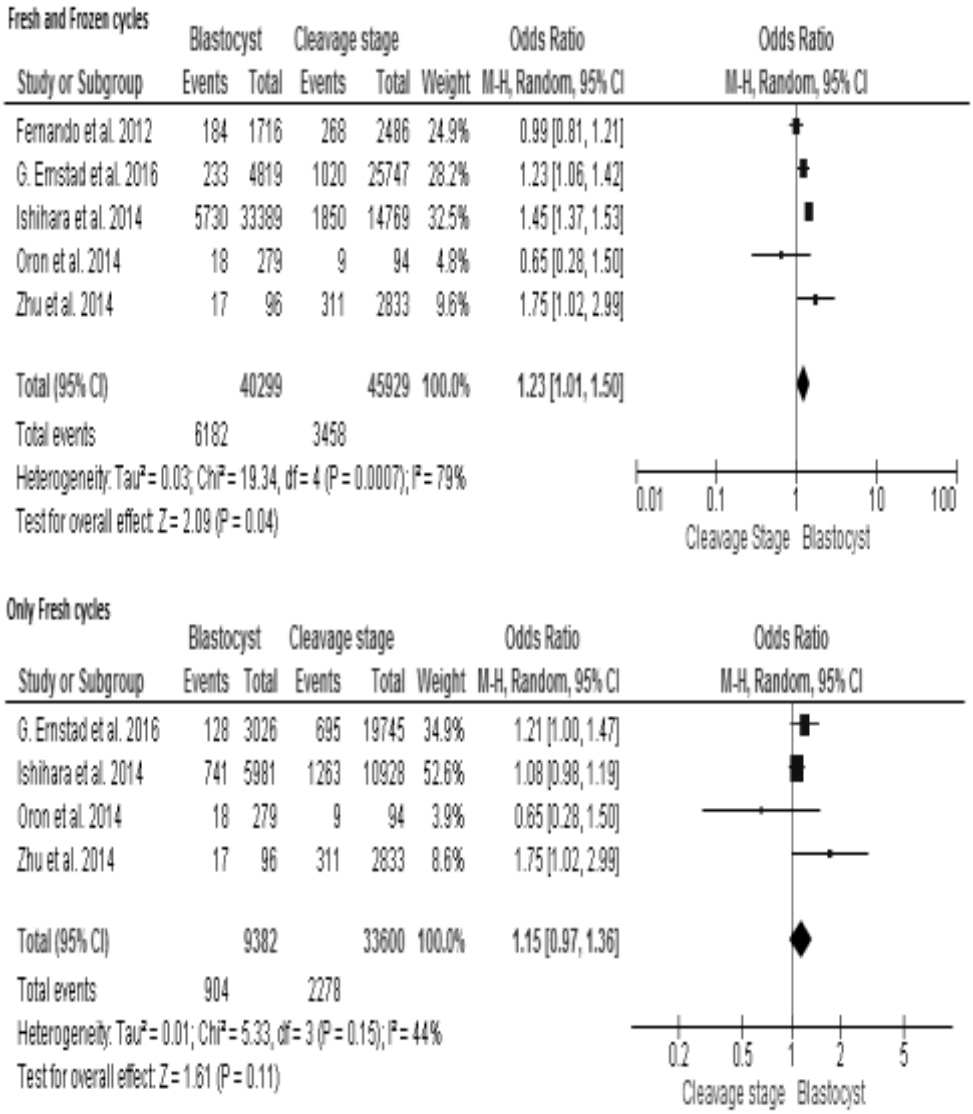
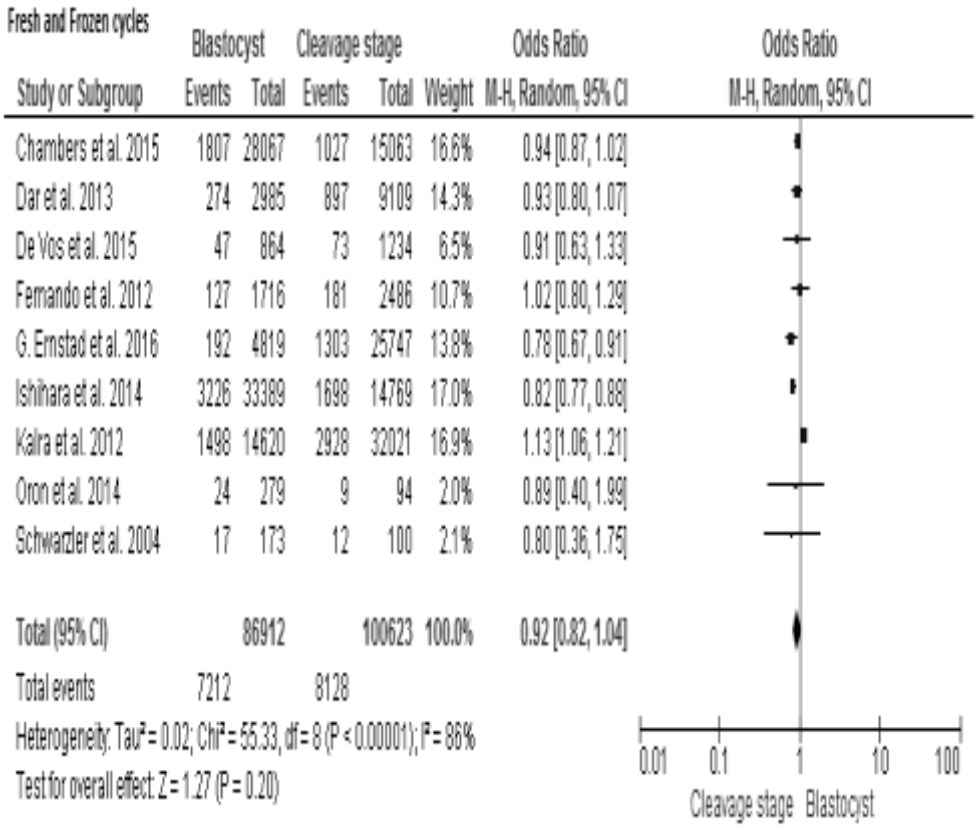
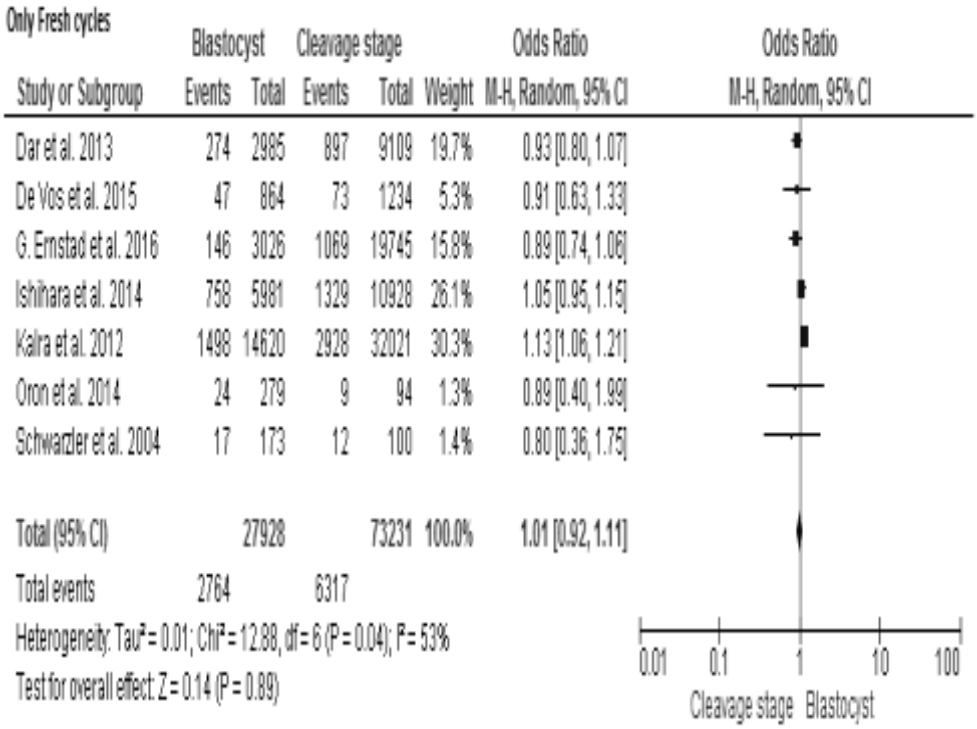


Fig. 5 Forrest-plot for LGA.

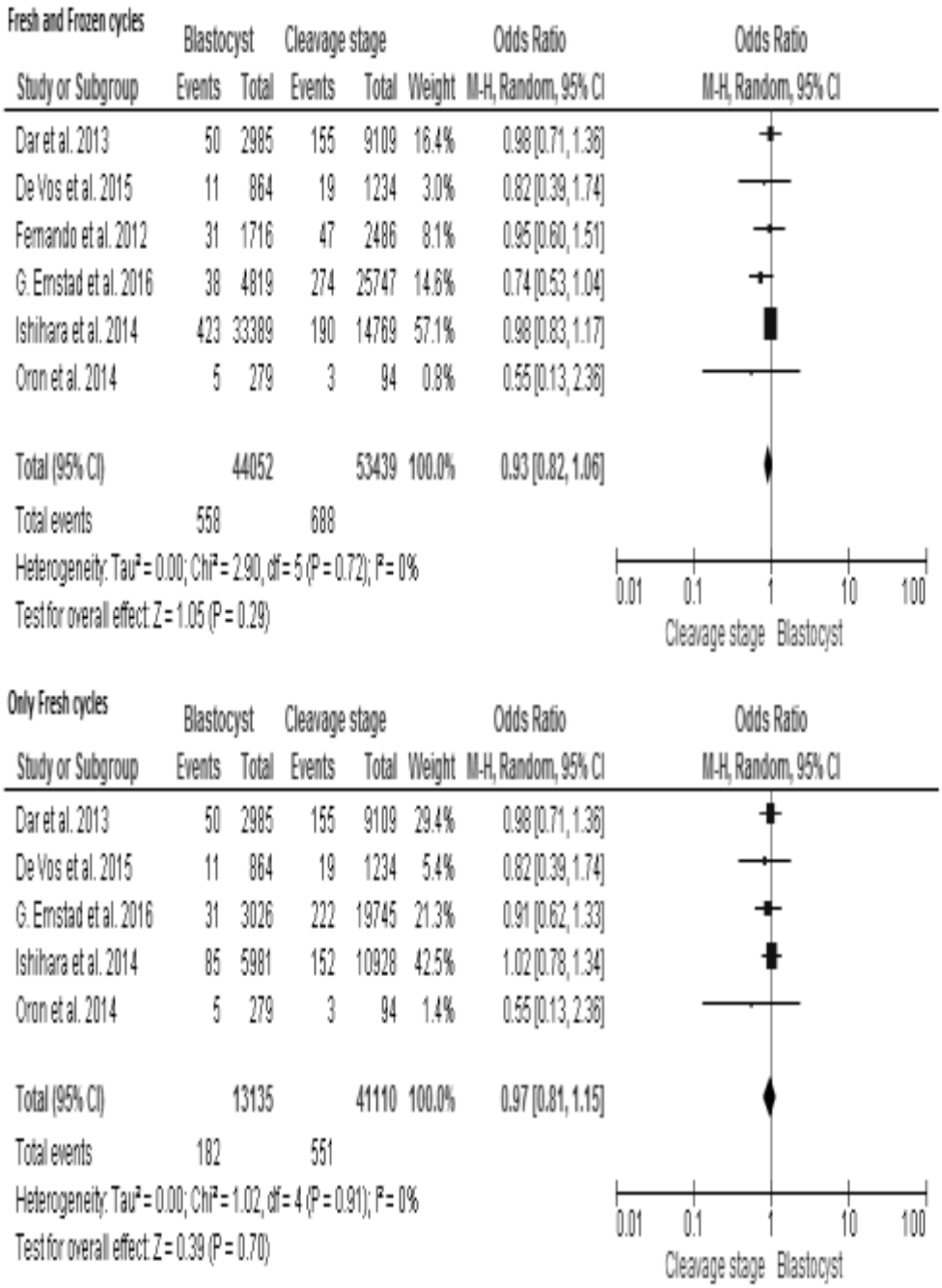


Supplemental data 1: Forrest-plot for low birth weight.

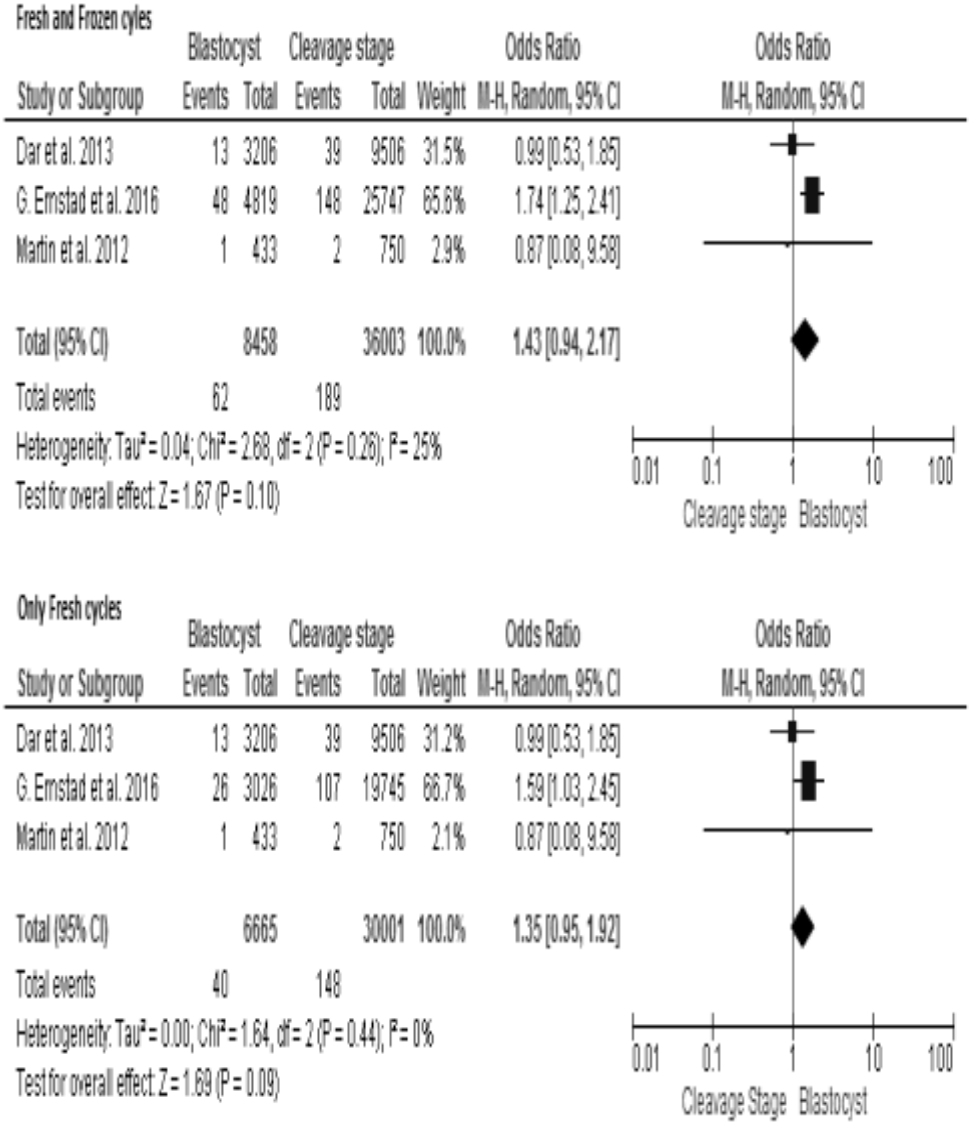




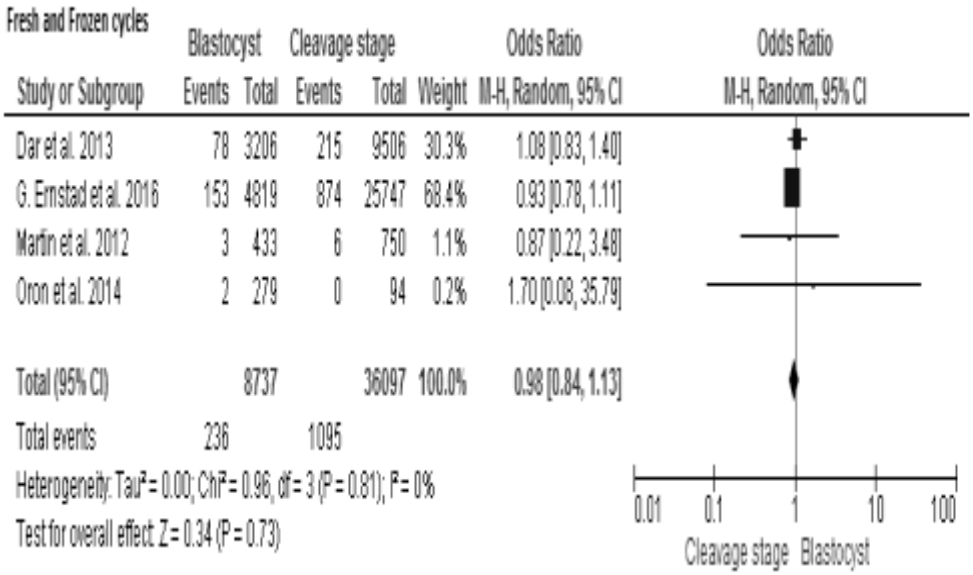
Supplemental data 2: Forrest-plot for very low birth weight.

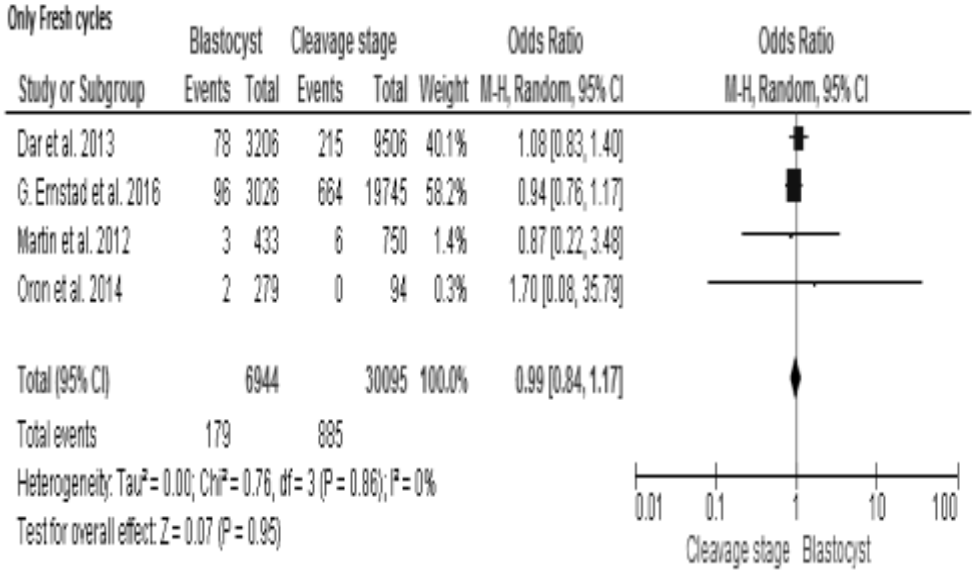


Supplemental data 3: Forrest-plot for perinatal mortal

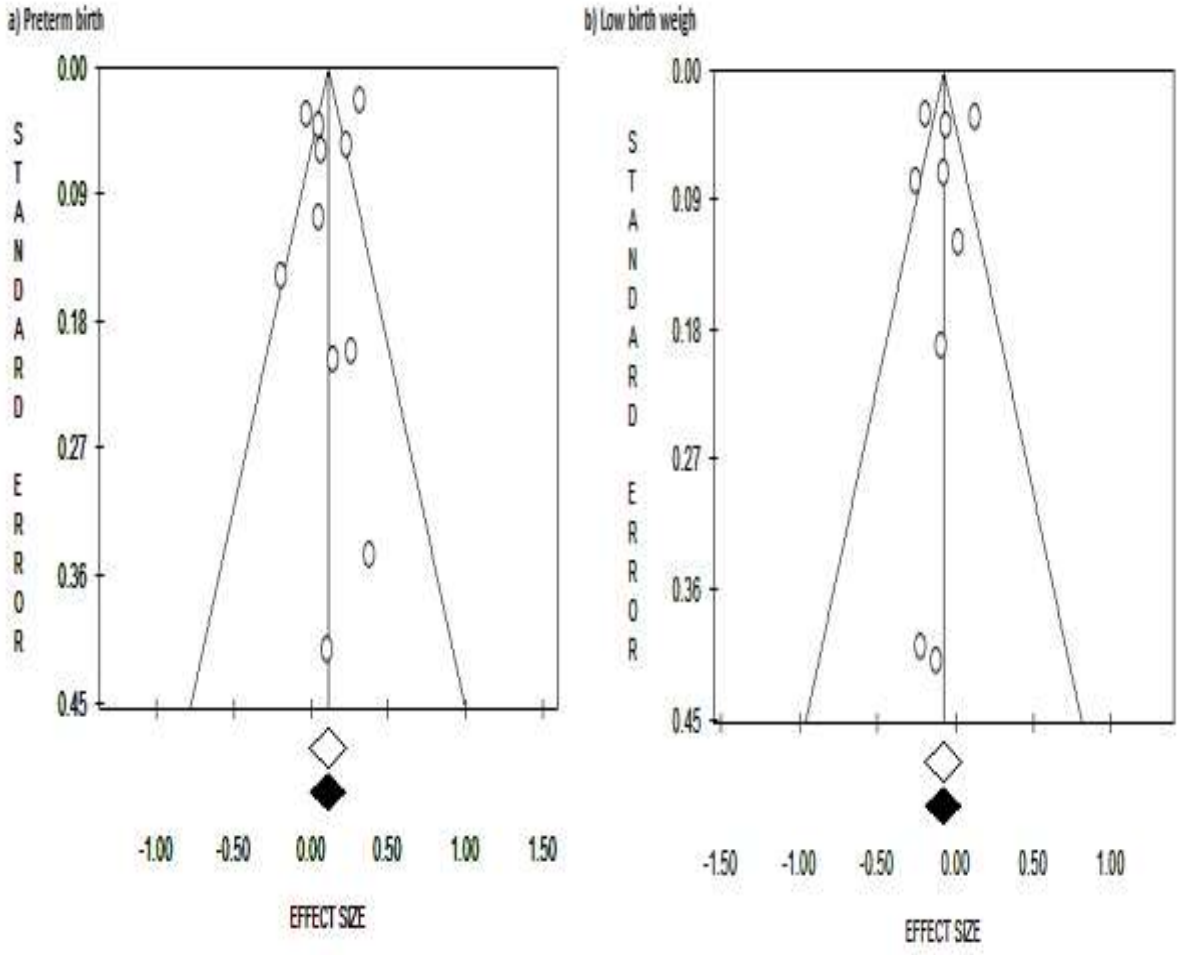


Supplemental data 4: Forrest-plot for congenital anomalies.





Supplemental data 5: Funnel plot and trim and firm analysis.



2. SYNOPSIS GENACOS

Clinical Trial Protocol Number	**GENACOS**
Short Title	**Impact of Gonadotropin GENetics Profile and OvArian Reserve on Controlled Ovarian Stimulation Outcomes**
Full Title	**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**
Trial Phase	**Investigator Sponsored Interventional non-pharmacological Trial**
EudraCT Number	
Coordinating Investigator& Sponsor	**Enrico Papaleo** **IRCCS San Raffaele Hospital, Milan, Italy**
Scientific Responsible	**Carlo Alviggi** **"University "Federico II", Naples, Italy**
Clinical centers	• **Centro Scienze della Natalità, *Ob-Gyn Dept.*, San Raffaele Hospital, Milan, Italy.** • **Dipartimento di Neuroscienze, Scienze Riproduttive ed Odontostomatologiche", University "Federico II, Naples, Italy** • **ANDROS Day Surgery Clinic, Reproductive Medicine Unit, Palermo, Italy**

	• **Clinica Zucchi - Biogenesi**
Centralized Laboratory	**Prof. Manuela Simoni** **Unit of Endocrinology, Department of Biomedical, Metabolic and Neural Sciences, University of Modena and Reggio Emilia, Italy** **Azienda USL of Modena, Italy**
Statistician	**Dr. Daniele Santi** **Unit of Endocrinology, Department of Biomedical, Metabolic and Neural Sciences, University of Modena and Reggio Emilia, Italy** **Azienda USL of Modena, Italy**
Scientific advisor	**Prof. Kypros Nicolaides** **King's College – London, UK**
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:** • **Dr Floriana Carbone, MD, PhD – Research Fellow-Fetal Medicine Foundation – UK**
Planned trial period (first subject in-last subject out)	**2 years of data collection**

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 all*, 2010), such as early-preeclampsia (Carbone *et all*, 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

≤AFC≤ 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 5rd 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 (≥ 2 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_2), LH and Progesterone (P_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 5th 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_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;

Eumenorroics normo-gonadotropic 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.

ExclusionCriteria:

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.

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