Assisted Reproduction Techniques; What's old and what's new

Panagakis G, Asimakopoulos G, Sfakianakis A, Theofanakis Ch, Kallianidis K, Drakakis P

Unit of Assisted Reproduction & Regenerative Medicine, 1st Department of Obstetrics & Gynecology, Alexandra Hospital, National & Kapodistrian University of Athens, Athens, Greece

Correspondence
Panagakis G, 76-78 Kafkasou str., 11363 Athens, E=mail: gpssas@yahoo.gr

Introduction

The greatest achievement of assisted reproductive medicine was the birth of Louise Brown in 1978. It was the result of many efforts by Edwards & Steptoe to achieve pregnancy with in vitro fertilization (IVF). Since then, there has been a rapid development of assisted reproduction techniques, with the ultimate aim of making infertility treatment more effective and affordable for most couples. The thorough study of reproductive mechanisms has led to a fuller understanding of the physiology and the pathology of fertilization, implantation and pregnancy. In fact, even in fertile couples, pregnancy expectancy ranges from 25-36%, including biochemical pregnancies.

The continued development of medical and diagnostic techniques has led to easier diagnosis of women with PCOS as well as those with a poor response to ovarian stimulation protocols. This has resulted in the individualization of treatment according to the needs and requirements of each underwhelmed woman. However, the increasing use of in vitro fertilization protocols has led to the development of theories of correlation with gynecological cancer.

Ovulation induction

Ovulation induction still remains one of the most challenging steps of reproductive medicine. Understanding the physiology of folliculogenesis, as well as the mechanisms by which the normal process of follicular selection may be overridden to produce multiple oocytes is essential in order to develop the optimal protocol for each patient.

Complete follicular development takes over 220 days and can be classified into three phases according to the developmental stage and the follicular gonadotropin dependence. The process begins with the initial recruitment of resting primordial follicles followed by the development of preantral and early antral follicles. Finally, cyclic recruitment of a limited cohort of antral follicles takes place, followed by the selection of a single dominant follicle during the mid-follicular phase of the menstrual cycle[1-3] In the adult ovary, folliculogenesis starts when follicles leave the pool of resting follicles to enter the growth phase. The size of the follicle pool is determined during fetal life and reaches its maximum of 6– 7 million by 20 weeks of gestation[4.] From this point on, germ cell content will decrease due to a continuous flow of follicles leaving the primordial follicle pool (initial recruitment). Around 1000 primordial

follicles start growing every month. However, the great majority of them will undergo atresia before reaching the antral stage, principally through apoptosis[3]. Only those follicles that are able to respond to stimulation by Follicle Stimulating Hormone (FSH) will enter the final stage of development and eventually ovulate.

During the follicular phase of the menstrual cycle in primates, a single follicle usually matures to the preovulatory stage and releases its oocyte for fertilization. Upon the demise of the corpus luteum, serum levels of FSH and LH increase (peri-menstrual rise) and the process of preovulatory follicular development is initiated. Brown et al. demonstrated that changes in FSH concentrations between 10–30% are sufficient to initiate follicular development in anovulatory women. Based on this finding, the concept of an "FSH threshold" was introduced, in order to indicate that a critical concentration of FSH must be achieved to initiate the process of follicular development[5]. The "FSH gate" or "FSH window" concept introduces the element of time rather than the magnitude of the FSH rise to the FSH threshold theory. The window concept emphasizes the importance of a transient increase of FSH above the threshold level in order to gain single dominant follicle selection[6, 7].

As already mentioned, a perimenstrual rise in FSH secretion occurs following the regression of the corpus luteum. Thereafter, there is a reciprocal relationship between the plasma concentrations of FSH and estradiol. During the early follicular phase prior to the emergence of a stimulated follicle, serum FSH concentrations are elevated while estradiol concentrations are low. Approximately five days before the midcycle gonadotropin surge, serum estrogen concentrations begin to rise as the result of the emergence of a maturing follicle. Associated with the gradual increase in estradiol concentrations is a progressive fall in FSH concentrations due to the feedback actions of estrogen (and possibly inhibin) on gonadotropin secretion[8, 9]. There always will be a maturational distinct population of early antral follicles within the ovaries ready for development to the preovulatory stage under the influence of FSH. As a growing follicle acquires sufficient aromatase activity, resulting from FSH stimulation, its production of estrogen suppresses FSH secretion below that level necessary to sustain the development of less mature follicles, which consequently undergo atresia.

Moreover, a hallmark action of FSH during preovulatory follicular development is the induction of LH receptors on granulosa cells[10]. Granulosa cells from early antral follicles possess FSH receptors and the stimulation of theese cells by FSH results in the activation of adenylyl-cyclase and the production of cAMP. In response to FSH stimulation, granulosa cells acquire LH receptors. The binding of the LH receptor by LH also results in the activation of adenylyl-cyclase and the production of cAMP[11]. As would be predicted by the common intracellular cAMP pathway, granulosa cells from FSH-stimulated follicles respond similarly to both FSH and LH; moreover, at low levels of FSH and LH, the responses are additive[12]. The overall significance of these findings is that while granulosa cells from early antral follicles are only responsive to FSH, granulosa cells from FSH-stimulated follicles are responsive to either FSH or LH. Thus it is possible that the maturing follicle reduces its dependence on FSH by acquiring LH receptors, hence LH responsiveness.

Ovalation is the main event of the menstrual cycle, as well as the dividing line between follicular and luteal phase. Levels of estradiol reach a threshold above which, this effect is reversed and estrogen stimulates the production of a large amount of LH. This process, known as the LH surge, starts around day 12 of the average cycle and may last 48 hours. Following the onset of the LH surge, a series of

events take place that lead to the rupture of the follicle[12].

Drugs in ovulation induction

Knowledge of the normal process of follicular selection allows for the understanding of the physiological principles that underlie various strategies for increasing the number of preovulatory follicles that can be stimulated to maturation. Controlled ovarian stimulation is achieved by increasing the duration that serum FSH concentrations are maintained above threshold levels. This can be achieved by direct administration of exogenous FSH. Alternately, administration of the anti-estrogens clomiphene citrate and tamoxifen as well administration of an aromatase inhibitor, in the presence or absence of exogenous FSH, also can result in ovarian stimulation presumably by diminishing the negative feedback effects of estrogen on FSH secretion[12-15]. The controlled ovarian stimulation during the IVF cycles is achieved by the dilution of the following drugs:

  i   Gonadotropins
  ii GnRH-analogues
  iii Clomiphene citrate

Gonadotropins

In the 65 years since the gonadotropin hormones were discovered, FSH has come to attain a central role in contemporary infertility therapies. Clinical applications include the treatment of anovulatory infertility and controlled ovarian stimulation in women being treated with IVF. At first, gonadotropins were extracted from urine of postmenopausal women (human menopausal gonadotropins) in a 1:1 ratio of LH and FSH. To improve batch-to-batch variability, non-active proteins were removed resulting in highly purified urinary preparations. The urinary extraction process needed large amounts of postmenopausal urine and the increasing demands for gonadotropins

compromised the possibilities to guarantee a consistent supply of the medication worldwide. When in the 1990s recombinant DNA technology made it possible to produce human FSH in Chinese hamster ovary cell lines, this production problem was solved.

Individual differences in the required daily amount of FSH to induce ongoing follicle growth (the FSH response dose) have been suggested to be the main factor in hyper-responsiveness and severe complications during gonadotropin ovulation induction[1]. This resulted in mainly two different approaches: the "step-up" and "step-down" protocols. A low-dose, step-up protocol designed to allow the FSH threshold to be reached gradually has now become the most widely used regimen, reducing the risk of excessive stimulation and development of multiple pre-ovulatory follicles. In this protocol, the initial subcutaneous or intramuscular dose of FSH is 50-75 IU/day; and the dose is increased if, after 14 days, no response is observed on ultrasonography (and serum estradiol monitoring). Increments of 37.5 IU are then given at weekly intervals up to a maximum of 225 IU/day. The detection of an ovarian response is an indication to continue the current dose until human chorionic gonadotropin (hCG) can be given to trigger ovulation.

Step-down protocols are aimed at rapidly achieving the FSH threshold for stimulating follicle development. Current regimens normally begin therapy with 150 IU/day started shortly after a spontaneous or progesterone-induced bleeding. This dose is continued until a dominant follicle (10 mm) is seen on transvaginal ultrasonography. The dose is then decreased to 112.5 IU per day followed by a further decrease to 75 IU per day 3 days later, which is continued until hCG is administered to induce ovulation. If no ovarian response is observed after 3-5 days, the FSH dose can be increased. For some patients, an initial dose of 150 IU/day may be too high, reflecting major individual differences in the

FSH threshold[5, 16-18].

GnRH-analogues (agonists, antagonists)

The main result following the administration of GnRH-agonists is that after an initial short period of gonadotropin hyper secretion, continuous administration causes desensitization, resulting in a state of chemical induced pituitary failure. There is also an initially increase in FSH and LH secretion (so-called 'flare effect'). After 10-14 days of continued administration, this results in down-regulation of the pituitary gland and the suppression of LH (about 70%) and FSH (about 30%) serum levels. GnRH agonists may be delivered by intranasal or subcutaneous formulations.

GnRH antagonists cause an immediate and rapid, reversible suppression of gonadotropin secretion by competitive occupancy of the GnRH receptor. The development of clinically safe agonists was relatively simple by just changing one or two amino acids. GnRH antagonists have been recently introduced in clinical practice for ovarian hyperstimulation in ART cycles to prevent premature luteinization[7, 19].

Clomiphene Citrate

Clomiphene citrate (CC) is the initial treatment for most anovulatory infertile women. Chemically similar to tamoxifen, CC is a nonsteroidal triphenylethylene derivative that demonstrates both estrogen agonist and antagonist properties. Antagonist properties predominate except at very low estrogen levels. As a result, negative feedback that is normally produced by estrogen in the hypothalamus is reduced. Gonadotropin releasing hormone (GnRH) secretion is altered and stimulates pituitary gonadotropin release. The resulting increase in follicle- stimulating hormone (FSH) levels, in turn, drives ovarian follicular activity. Clomiphene therapy for ovulation induction is typically started on the fifth day of a cycle, following either spontaneous or induced bleeding. It

is initially begun at a dose of 50 mg daily for 5 days. If ovulation does not occur in the first cycle of treatment, the dose is increased to 100 mg. Thereafter, dosage is increased by increments of 50 mg to a maximum daily dose of 250 mg. However, a dose of more than 150 mg is not encouraged. Drug-induced side effects are hot flushes (occurring in 10-20% of women), abdominal distention and pain (5.5%), nausea and vomiting (2.2%) and breast discomfort (2%)[20, 21].

Aromatase Inhibitors

Blocking estrogen production by inhibiting the enzyme catalyzing its synthesis from androgens (aromatase enzyme) is a treatment modality that has been in clinical application for more than half a century since the development of the first generation of aromatase inhibitors including aminoglutethimide. Aromatase inhibitors are orally administered, easy to use, relatively inexpensive, and associated with typically minor side effects. At doses of 1-5 mg/day, these drugs inhibit estrogen levels up to 99%. The most widely used aromatase inhibitor to induce ovulation in anovulatory and ovulatory infertile women is letrozole. A second aromatase inhibitor, anastrozole, is of the same compound class as letrozole and has also been approved for treatment of women with breast cancer. There is no significant difference in the use of letrozole or anastrozole in pregnancy rates or miscarriage rates when used for ovulation induction in women with clomiphene-resistant PCOS. However, these medications are used "off label" regarding assisted reproduction. The main side effects are hot flushes, gastrointestinal events (nausea and vomiting), headache, back pain and leg cramps[22, 23].

Controlled ovarian stimulation protocols

Controlled ovarian stimulation protocols using GnRH analogues (agonists /antagonists) aim to achieve gonadotropin secretion. The regimens

using GnRH- agonists are the long, short and ultra short protocol. The protocols using GnRH-antagonists are those in which we administrate one single dose of the antagonist (rarely used) those in which we administrate multiple doses.

Long protocol

It is the most often used protocol in order to achieve multiple ovulation in IVF, based on the secondary action of GnRH in the pituitary gland. The administration of the analogue starts at the beginning of the cycle or at the end of the previous one, for at least 7 days prior to the initial administration of the gonadotropins. Serum estradiol <40pg / ml indicates pituitary suppression. The risk of impulsive LH-surge is reduced as well as the risk of premature ovulation. After the achievement of suppression, administration of gonadotropins begins daily. It is essential to measure estradiol and to monitor by ultrasound the follicular maturation after each administration. According to ultrasound image of 3 or more follicles of at least 17mm diameter hCG is administrated to trigger ovulation. Egg retrieval is scheduled 36 hours later and then IVF is performed in a laboratory setting. Embryos are transferred back to the uterus 3–5 days following retrieval. Progesterone supplementation, with either vaginal preparations or intramuscular injection, follows for luteal phase support. Often, pretreatment with combined oral contraceptives (COCs) is preferred, in order to prevent ovarian cyst formation[24].

Short protocol

This is also known as the flare protocol. GnRH agonists initially bind gonadotropes and stimulate follicle-stimulating hormone (FSH) and LH release. This initial flare of gonadotropes stimulates follicular development. Following this initial surge of gonadotropins, the GnRH agonist causes receptor downregulation and an ultimately hypogonadotropic state. The administration of the agonist starts from day 1 to day 2 of the cycle and gonadotropin injections begin 2 days later, to resume follicular development. Same as in the long protocol, continued GnRH-agonist therapy prevents premature ovulation. However, this protocol is characterized by a lower percentage of clinical pregnancies compared to the Long protocol[24].

Ultra-short Protocol

It is a variation of the short protocol, in which the analogue is administered for 3 days, in order to achieve ovarian stimulation, followed by a course of gonadotropins. In this protocol, the early peak of LH appears more often and, therefore, it leads to poor results. It is usually used in poor responders, in order to achieve more intense stimulation of the ovaries[24].

GnRH-antagonist protocol

As with GnRH agonists, these agents are combined with gonadotropins to prevent premature LH surge and spontaneous ovulation. This protocol attempts to minimize the risk of ovarian hyperstimulation syndrome (OHSS) and GnRH side effects, such as hot flashes, headaches, bleeding, and mood changes. The multiple dose protocol consists of the daily administration of the antagonist from day 6 of the follicular phase along with gonadotropin administration. When the dominant follicle reaches the diameter of 18-20mm, the antagonist is stopped and ovulation is induced by hCG. The single dose protocol is rarely used. In GnRH-antagonists protocol with corifollitropin-a (100-150μg), the administration of corifollitropin starts at the 2nd day of cycle, the antagonist's daily from the 6th day and, if necessary, gonadotropins are administrated daily from the 8th day. Following ovulation, the procedure is the same as in the agonist protocols[7, 25].

Polycystic Ovary Syndrome

Polycystic Ovary Syndrome (PCOS) is one of the most common endocrinopathies, affecting 5 – 10 % of women of reproductive age[26]. The syndrome is characterized by a wide range of endocrine and metabolic disorders, such as hyperandrogenism, anovulation, insulin resistance, dyslipidemia, central obesity – metabolic syndrome. In the long term, it may increase the risk of diabetes mellitus and cardiovascular disease[26]. Another common issue of women with PCOS is infertility related to the ovulation disorders of the syndrome.

The syndrome was surrounded by controversies regarding its diagnosis and treatment. The need for universally accepted diagnostic criteria led to the Rotterdam meeting in 2003, during which experts in PCOS arrived at a consensus regarding the diagnosis of the syndrome[27] Therefore, two out of the following three criteria should be present for the diagnosis of PCOS :

1) Oligo- or anovulation,
2) Clinical and/or biochemical signs of hyperandrogenism,
3) Polycystic ovaries (>12 follicles 2-9mm or ovary volume > 10 ml)

Other etiologies of hyperandrogenism and ovulation disorders, such as congenital adrenal hyperplasia, androgen-secreting tumors, Cushing 's syndrome, hyperprolactinemia and thyroid disorders, should be excluded[27].

Women with PCOS are characterized by infertility issues, affecting almost 70–80% of those women. According to the American Society of Reproductive Medicine, women with PCOS should be investigated for infertility issues after six months of unprotected intercourse without conception[28].

The infertility treatment options include initially non-pharmacological and then pharmacological options. The choice of the most appropriate treatment depends on the patient 's age, existence of other in-fertility factors, previous treatments and the level of anxiety of the couple[29].

Non-pharmacological options

Among non-pharmacological treatments, lifestyle modifications, such as diet and exercise aiming to weight loss and smoking/alcohol cessation, are the first-line treatment option for infertile obese women with PCOS. A weight loss of 5–10 % over a period of six months may improve hyperadrogenism, central obesity and ovulation rate[27]. However, there is not enough evidence showing improvement of live birth rate. There are several randomized trials demonstrating the positive effect of weight loss on ovulation rate and hyperandrogenism in women with PCOS[30-34].

Pharmacological options

The pharmacological treatment options include clomiphene citrate as first-line pharmacological treatment, gonadotropins and ovarian drilling as second-line treatment and in vitro fertilization as third-line treatment. Other treatment options include metformin and aromatase inhibitors.

Clomiphene citrate

Clomiphene citrate (CC) is an estrogen receptor modulator which binds to estrogen receptors in the hypothalamus and pituitary and block the negative feedback mechanism. Consequently, the levels of releasing gonadotropins are increased leading to the recruitment of the dominant follicle between the 6th and 9th day of the menstrual cycle[35]. Clomiphene citrate is the first choice for ovulation induction for anovulatory women with PCOS[27, 36].

The predictive factors of live birth after clomiphene citrate induction of ovulation in normogonadotropic oligoamenorrheic infertility include patient 's age, Body Mass Index (BMI) and Free Androgen Index (FAI)[35]. The initial dose is 50

mg/day for five days, starting between the second and fifth day of the menstrual cycle (spontaneous or progestagen-induced). Almost 50% of pregnancies are achieved with this dose. Ovulation is expected 8-10 days after the end of the five day CC administration, but it may be delayed up to 2 - 3 weeks. Unless normal ovulation occurs, the dose is increased by 50 mg/day in each of the next cycles up to a maximum dose of 150 mg/day[18]. However, doses greater than 100 mg/day usually do not offer additional benefits (may be useful in obese women)[21]. The ovulation rate after CC induction is almost 75 – 80% [20], the conception rate is estimated about 22% per cycle[37], whereas the cumulative pregnancy rate is almost 60–70% after six cycles[38].

The duration of the treatment should be limited up to 6 ovulatory cycles. Additional cycles of induction with CC (maximum of twelve cycles) should be individually evaluated based on the age of woman and after discussion with the couple, whereas second-line treatment options (gonadotropins, ovarian drilling) should be considered[27].

About 15-40% of women with PCOS do not respond to 150mg/day of CC for 5 days/cycle for at least 3 cycles and are considered clomiphene resistant. The risk factors for CC resistance include insulin resistance, hyperandrogenism and obesity which disrupt ovary response to the increased levels of FSH after induction with CC[39]. Furthermore, the presence of specific genetic background may be another risk factor for CC resistance. Overbeek et al [40] demonstrated that Ser680Ser FSHR gene polymorphism is associated with higher, almost double, risk for CC resistance compared to Asn680Asn and Asn680Ser FSHR gene polymorphisms. Failure to conceive after 6-9 cycles of regular ovulation with CC induction is defined as CC failure.

CC treatment is characterized by advantages such as oral administration, low cost, few side effects (headache, flushes and visual disturbances), low rate of OHSS (1 – 6%) [29] and low rate of multiple gestation (6 – 8%)[18].

The observed difference between the ovulation rate and the lower pregnancy rate after treatment with CC is attributed to the anti-estrogenic effect of CC on peripheral organs such as endometrium and cervix. Specifically, the anti-estrogenic effect of CC on cervical mucus characteristics disrupts sperm penetration. Moreover, the anti-estrogenic effect of CC on endometrial proliferation disrupts embryo implantation. If the endometrial thickness is less than 8mm at the time of ovulation, poor prognosis is expected. However, Kolibianakis et al. [41] observed that endometrial thickness can not predict ongoing pregnancy achievement in cycles stimulated with clomiphene citrate for intrauterine insemination.

Gonadotropins

Gonadotropins are recommended as second-line treatment for PCOS related infertility issues. This option includes the use of recombinant FSH (rFSH) or Human Menopausal Gonadotropin (HMG). According to the threshold concept, the initiation and maintenance of follicle growth may be achieved by a transient increase in FSH above a threshold dose for sufficient duration to generate a limited number of developing follicles. This concept has essential role on women with PCOS who undergo ovulation induction due to their tendency to develop excessive number of follicles[27].

The complexity of that treatment makes necessary the evaluation of tubal patency prior to the initiation of ovulation induction with gonadotropins[42]. The ovarian stimulation begins with low doses of gonadotropins (37.5-75 IU/day or every other day), in order to achieve oligofollicular growth and decrease the risk of OHSS and multiple gestation[43]. Currently two low-dose regimens are utilized, step-up regimen and step-down regimen, which are characterized by similar rates of oligofollicular

growth[44]. However, Christin-Maitre et al[45] observed that step-up regimen is more safe regarding the risk of excessive multiple follicle growth. Moreover, the application of a step-down regimen requires more experience and ability to adjust the gonadotropin dose compared to a step-up regimen[46]. The use of a low-dose regimen achieves a rate of oligofollicular growth at about 70%, a pregnancy rate at 20% [47], a rate of OHSS lower than 6% and a rate of multiple gestation lower than 1%[48]. Eijkemans et al[49] demonstrated that the cumulative pregnancy rate resulting in singleton live birth of a consecutive series of 240 normogonadotrophic anovulatory infertile women undergoing classical ovulation induction (CC as first-line, followed by FSH as second-line therapy if required) was almost 72%.

Ovarian drilling

Apart from the pharmaceutical second-line approach with gonadotropins, ovarian drilling offers an additional second-line invasive treatment for PCOS infertility issues. The effect of ovarian drilling seems to be based on the destruction of the androgen-producing ovarian stroma and follicles leading to a reduced secretion of androgens and inhibin and a consequent increase of FSH secretion[50]. Furthermore, the thermal damage results in the production of inflammatory factors (e.g. IGF-1), which enhance FSH action on folliculogenesis[51]. Also, the surgical intervention on ovary facilitates ovarian blood flow leading to an increased transfer of gonadotropins[52]. This technique includes monopolar diathermy or laser without any obvious difference in outcomes between the two modalities[27]. The number of punctures is usually 4 -10 for each ovary depending on the ovarian size. Most surgeons recommend the lowest effective dose of four punctures per ovary, each for 4 seconds at 40 W (rule of 4), delivering 640 J of energy per ovary[51]. Higher number of punctures increases the risk of premature ovarian failure

and adhesions, which may have a negative influence on fertility at reproductive age[53].

Due to the invasive nature of this procedure, the high cost, the need for general anesthesia and hospitalization and the high risk of complications, this technique should be used in specific cases of anovulatory women of normal BMI with CC-resistant PCOS who require laparoscopy for another reason (pelvic pain, adnexal mass, etc.) or live too far away from the hospital for the intensive monitoring required during gonadotropin therapy. Laparoscopic ovarian drilling can also be used to women with hypersecretion of luteinizing hormone (LH) during natural cycles or as a response to CC[27, 54-56]. This procedure should not be offered for non-fertility indications, such as menstrual irregularity, metabolic complications or hyperandrogenism in PCOS[57].

The ovulation rate ranges between 54 and 76% in 6 months after the procedure and 33 and 88% in 12 months after the procedure. The respective spontaneous pregnancy rates range between 28 and 56% for 6 months and 54 and 70% for 12 months[58]. In case of failing to induce ovulation at 3 months after ovarian drilling, the procedure should be combined with CC. The use of gonadotropins should be considered after 6 months of anovulation following the procedure. Amer et al[59] demonstrated no statistical difference of cumulative pregnancy rates [P = 0.26, OR 1.6 (0.6–4.2)] between laparoscopic ovarian drilling (52%) and CC (63%) at 12 months follow-up, when each of these treatments was used as a first-line method of ovulation induction in women with polycystic ovary syndrome. Consequently, laparoscopic ovarian drilling is not superior to CC as a first-line method of ovarian induction in women with PCOS. Farquhar et al[57] showed no statistical difference in live birth rate between laparoscopic ovarian drilling and the combination of CC and tamoxifen (OR 0.81; 95% CI 0.42 to 1.53; P = 0.51) or gonadotropins (OR 0.97; 95%

CI 0.59 to 1.59; P = 0.89) or aromatase inhibitors (OR 0.84; 95% CI 0.54 to 1.31; P = 0.44). However, laparoscopic ovarian drilling was found to be associated with a lower live birth rate compared to the combination of CC and metformin (OR 0.44; 95% CI 0.24 to 0.82; P = 0.01) and a lower rate of multiple gestation compared to gonadotropins (OR 0.13; 95% CI 0.03 to 0.52; P=0.004). Ovarian drilling is characterized by the same effectiveness as gonadotropins regarding the pregnancy rate and the live birth rate. However, this technique does not require intensive monitoring of the patient and is associated with lower risk of multiple gestation and no risk of OHSS in comparison to gonadotropins[60].

In Vitro Fertilization (IVF)

In Vitro Fertilization is recommended as third-line therapeutic approach for infertile women with PCOS. However, in case of bilateral tubal occlusion or concentration of recovered motile sperm less than or equal to 5 million, IVF becomes the first fertility option in combination with lifestyle modifications.

The main complication of this approach is OHSS. In order to reduce the risk of OHSS, ovarian stimulation should be initiated with low doses of gonadotropins (100 – 150 IU rFSH) and the pituitary suppression should be accomplished with a GnRH antagonist, which is associated with a lower risk of OHSS related to GnRH agonists[19, 61].

A metaanalysis demonstrated that the use of conventional IVF protocol to women with PCOS is associated with a higher number of retrieved oocytes per cycle, a lower fertilization rate and a higher cancellation rate compared to normo-ovulatory women without PCOS. However, PCOS and control patients achieved similar pregnancy [OR = 1.0 (95% CI 0.8–1.3)] and live birth [OR = 1.0 (95% CI 0.7–1.5)] rates per cycle[62]. Another metanalysis showed no difference in pregnancy rate per transferred embryo between GnRH antagonists and long

protocol with GnRH agonists (RR 0.97; 95% CI 0.85–1.10). However, the use of GnRH antagonists was found to be associated with a lower risk of severe OHSS (OR 1.56; 95% CI 0.29–8.51)[25].

Other treatment options
Metformin

Metformin is an insulin sensitizing agent with a multi-target mechanism of action. It has been shown to improve systemic insulin resistance, serum androgen levels, ovarian hyperadrogenism through local effect on ovarian steroidogenesis, intra-ovarian insulin resistance and endometrial receptivity. According to various protocols, the recommended dose of metformin is 1500–1700 mg per day, starting 4–14 weeks prior to ovarian stimulation until ovulation triggering or pregnancy test.

Tang et al[63] demonstrated that metformin was associated with improved clinical pregnancy rates compared to placebo (pooled OR 2.31, 95% CI 1.52 to 3.51, 8 trials, 707 women). Improved clinical pregnancy rates were also found for the combination of metformin and clomiphene compared to clomiphene alone (pooled OR 1.51, 95% CI 1.17 to 1.96, 11 trials, 1208 women). However, there was no evidence that metformin improved live birth rates, whether it was used alone (pooled OR 1.80, 95% CI 0.52 to 6.16, 3 trials, 115 women) or in combination with clomiphene (pooled OR 1.16, 95% CI 0.85 to 1.56, 7 trials, 907 women).

Regarding the administration of metformin to patients with PCOS who receive gonadotropins for ovulation induction and timed intercourse (TI) or intrauterine insemination (IUI), a systematic review with meta-analysis of randomized controlled trials [64] revealed almost double pregnancy (OR = 2.25, 95% CI 1.50 to 3.38; P < 0.0001) and live birth (OR = 1.94, 95% CI 1.10 to 3.44; P = 0.020) rates without significant heterogeneity across the studies (P = 0.710, estimation of inconsistency = 0% and P

= 0.230, estimation of inconsistency = 30%; respectively, for pregnancy and live-birth rates). Furthermore, the metformin administration was associated with reduced cancellation rate (OR = 0.41, 95% CI 0.24 to 0.72, P = 0.002), stimulation length (MD = -3.28, 95% CI -6.23 to 0.32, P = 0.030) and gonadotropin dose (MD = -306.62, 95% CI -500.02 to -113.22, P = 0.002). No statistical difference was found regarding the multiple pregnancy rate (OR = 0.32, 95% CI 0.08 to 1.23; P = 0.100), the miscarriage rate (OR = 0.47, 95% CI 0.14 to 1.54; P = 0.210) and the OHSS rate (OR = 0.56, 95% CI 0.26 to 1.21; P=0.140).

Another systematic review and meta-analysis of randomized controlled trials [65] investigated the effects of metformin in patients with PCOS treated with gonadotropins for assisted reproduction (IVF/ICSI) cycles and demonstrated no significant improvement on clinical pregnancy (OR 1.20, 95% CI 0.90–1.61, P = 0.253) or live birth (OR 1.69, 95% CI 0.85–3.34, P = 0.132) rates. However, metformin seems to reduce the risk of OHSS (OR 0.27, 95% CI 0.16–0.46, P < 0.0001) and improve the rates of miscarriage (OR 0.50, 95% CI 0.30–0.83, P = 0.010) and implantation (OR 1.42, 95% CI 1.24–2.75, P = 0.040). On the contrary, Tso et al [66] demonstrated improvement of clinical pregnancy rates (OR 1.52; 95% CI 1.07 to 2.15; eight RCTs, 775 women, I(2) = 18%, moderate-quality evidence) with metformin administration before and during IVF or ICSI in women with PCOS, but without clear evidence of improvement of live birth rates (OR 1.39, 95% CI 0.81 to 2.40, five RCTs, 551 women, I(2) = 52%, low-quality evidence).

Given that the use of a GnRH antagonist in combination with gonadotropins for ovarian stimulation in PCOS patients and the final maturation induction with a GnRH agonist, followed by embryo cryopreservation, are considered as more effective strategies for the prevention of OHSS regardless of

metformin use[61], the routine use of metformin in IVF cycles in patients with PCOS is not recommended except in the presence of glucose metabolism disorder[27].

Aromatase Inhibitors

The mechanism of action of aromatase inhibitors is based on the reduced synthesis of estrogens from androgens at the ovarian granulosa cells by inhibiting the action of the enzyme aromatase, leading to reduced serum estrogen levels. Therefore, aromatase inhibitors block the negative feedback mechanism, resulting in increased levels of released gonadotropins[22]. Aromatase inhibitors have also been used for the prevention of anti-estrogenic effect of CC on endometrium. The recommended dose is 2,5–7,5 mg per day for 5 days from day 3 to day 7 of the cycle.

Misso et al[22] demonstrated that letrozole is associated with higher ovulation rate per patient compared to CC [OR 2.90 (95% CI 1.72, 4.88), I2 = 0%, P = 0.0001], without achieving significant improvement of pregnancy rate [OR 1.53 (95% CI 0.91, 2.58), I2 = 50%, P = 0.11], live birth rate [OR 0.48 (95% CI 0.07, 3.55), I2 = 0%, P = 0.48], multiple pregnancy rate [OR 2.53 (95% CI 0.53, 12.16), I2 = 0%, P = 0.25] and miscarriage rate [OR 0.66 (95% CI 0.22, 1.95), I2 = 0%, P = 0.45]. However, for cycles followed by timed intercourse, letrozole seems to improve pregnancy (fifteen studies; OR 1.40, 95% CI 1.18 to 1.65, n=2816, I^2=26%) and live birth (nine studies; OR 1.64, 95% CI 1.32 to 2.04, n=1783, I^2=3%) rates in subfertile women with anovulatory PCOS, compared to clomiphene citrate. In comparison to ovarian drilling, letrozole shows no difference in effectiveness regarding the pregnancy rate (OR 1.14, 95% CI 0.80 to 1.65, n = 553, I^2 = 0%), the live birth rate (OR 1.19, 95% CI 0.76 to 1.86, n = 407, I^2 = 0%) and the OHSS rate[67]. Legro et al[68] resulted in the same conclusions for cycles followed

by timed intercourse after the administration of letrozole or CC. The cumulative ovulation rate was higher with letrozole than with clomiphene (834 of 1352 treatment cycles-61.7% vs. 688 of 1425 treatment cycles-48.3%, P<0.001). The cumulative live birth rate was also higher for letrozole group than clomiphene group (103 of 374 [27.5%] vs. 72 of 376 [19.1%], P = 0.007; rate ratio for live birth, 1.44; 95% confidence interval, 1.10 to 1.87) without significant differences in overall congenital anomalies (P = 0.65). There were no significant differences between letrozole group and clomiphene group in pregnancy loss rates (49 of 154 pregnancies in the letrozole group [31.8%] and 30 of 103 pregnancies in the clomiphene group [29.1%]) or twin pregnancy rates (3.4% and 7.4%, respectively).

Aromatase inhibitors could be an option before IVF/ICSI in special cases of PCOS patients resistant to CC without any other infertility factor, for whom the application of high-complexity treatments is cost-prohibitive[22].

Poor responders

In 1983, Garcia et al. decribed for the first time a patient with a poor response in ovarian stimulation. The patient presented had a decreased follicular response and low estradiol levels to ovarian stimulation by FSH/HMG. This resulted in few oocytes being retrieved and few transferred embryos[69]. Since then, many studies have been conducted in an effort to characterize and find the optimal possible treatment for this category of infertile patients[70-72]. However, despite the ongoing interest for this group of patients, there was insufficient evidence for researchers to provide with a proper universal definition of ovarian poor response. Based on that fact, in 2011 a consensus was organized by the European Society of Human Reproduction and Embryology (ESHRE) with the purpose to analyze the

existing data and deliver a definition for the optimal description of poor responders, which resulted to the Bologna criteria[73]. At least two of the following three features must be present, in order to define a poor ovarian response (POR):
i. Advanced maternal age (≥40 years) or any other risk factor for POR;
ii. A previous POR (≤ 3 oocytes with a conventional stimulation protocol);
iii. An abnormal ovarian reserve test (i.e. AFC < 5-7 follicles or AMH < 0.5-1.1 ng/ml)

In order to define a patient as poor responder, two episodes of POR after maximal stimulation are sufficient, in the absence of advanced maternal age or abnormal ovarian reserve test (ORT). By definition, the term POR refers to the ovarian response, thus one stimulated cycle is considered essential for the diagnosis of POR. The consensus stated that patients over 40 years of age with an abnormal ORT may be classified as poor responders, since both advanced maternal age and an abnormal ORT cloud indicate reduced ovarian reserve and act as surrogate of ovarian stimulation cycle. In this case, the patients should be more properly defined as expected PORs[73].

A few years ago, a double protocol was tested, aiming to optimize the ovarian stimulation in poor responders, providing with an alternative regime for this group of patients. This was known as the Shanghai protocol and it was designed to investigate the efficacy of double stimulations during both the follicular and luteal phases in patients with poor ovarian response undergoing IVF and intracytoplasmic sperm injection (ICSI) treatments. At the first stage, clomiphene citrate 25 mg/day co-treatment and letrozole 2.5 mg/day is given from cycle day 3 onwards. Letrozole is only given for 4 days and clomiphene citrate is continuously used before the trigger day. Patients start to inject human menopausal gonadotropin (HMG) 150 IU every

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other day beginning on cycle day 6. Follicular monitoring starts on cycle day 10 and is carried out every 2–4 days using a transvaginal ultrasound to record the number of developing follicles and serum FSH, LH, estradiol and progesterone concentrations. When one or two dominant follicles reach 18 mm in diameter, the final stage of oocyte maturation is induced with triptorelin, followed by ibuprofen, which is used on the triggering day and the next day, for preventing possible follicle rupture before oocyte retrieval. Transvaginal ultrasound-guided oocyte retrieval is conducted 32–36 h after GnRH agonist administration. Follicles of less than 10 mm are not retrieved and left for the second-stage stimulation in the luteal phase. At the second stage, transvaginal ultrasound examination is carried out after oocyte retrieval to determine whether to proceed to the second ovarian stimulation. The criterion for continued stimulation is the presence of at least two antral follicles 2–8 mm in diameter. A total of 225 IU HMG and letrozole 2.5 mg is administered daily from the day of, or the day after, oocyte retrieval. The initial second stage follicular monitoring is conducted 5–7 days later, and then every 2–4 days, using a transvaginal ultrasound examination to record the number of developing follicles, and serum FSH, LH, estradiol and progesterone concentrations. Letrozole administration is discontinued when the dominant follicles reach diameters of 12 mm, given that large follicles have redundant LH and FSH receptors, and good response to exogenous hormone stimulations. Daily administration of medroxyprogesterone acetate 10 mg is added beginning on stimulation day 12 for cases in which post-ovulation follicle size is smaller than 14 mm in diameter and stimulation needed to continue for several more days. The purpose is to postpone menstruation and avoid oocyte retrieval during menstruation and also to prevent the risk of infection from the procedure. When three dominant follicles reach 18 mm or one

mature dominant follicle exceeds 20 mm, the final stage of oocyte maturation is induced again with triptorelin by injection. Again, ibuprofen is used on the day of oocyte maturation triggering and the day after. Transvaginal ultrasound-guided oocyte retrieval is conducted 36–38 h after GnRH agonist administration. After retrieval, the procedure at both stages follows the same methodology as in the agonist protocols[74].

Assisted reproduction and carcinogenesis

With approximately 1 million in vitro fertilization cycles reported per year worldwide, it seems of great importance to evaluate any potential association between exposure to fertility treatments and carcinogenesis[75]. Over the last decades, a plethora of studies have been published, regarding the long-term impact of ovulation inducing drugs on cancer risk. The main malignancies evaluated were ovarian, endometrial, breast and cervical cancer. Non-gynecological malignancies have also been evaluated[76]. The majority of the studies failed to reach to definite conclusions and despite the fact that a link between fertility drugs and cancer development is potentially suggested, results are extremely difficult to interpret. This is mainly because of the limitations on the methodology of the epidemiological studies, including the heterogeneity of the groups studied, with underlying diagnoses that are independently associated with carcinogenesis, such as anovulation, PCOS and endometriosis. Moreover there is a difficulty in achieving a long term follow up in these patients and this makes the establishment of a robust etiopathogenetic association between fertility treatments and cancer, extremely problematic. Furthermore, current scientific evidence demonstrates that infertile and nulliparous women are independently associated with an increased risk of developing ovarian, endometrial and breast cancer[77, 78].

When considering the relationship between fer-

tility drugs and ovarian cancer, several potential theories have been suggested regarding the pathogenesis of the disease. Nulliparity itself represents a risk factor, based on the theory of the "incessant ovulation", which suggests that prolonged and uninterrupted years of ovulation increase cancer risk[79]. The basic idea behind this theory is that a dysfunction in the mechanism for the recognition and repair of DNA damage, at the site of ovulation, is likely to be the initial step in ovarian tumor genesis[80]. This is supported by the observations that the risk for ovarian cancer in gravid women and women who have utilized chronic ovarian suppression is decreased. Other studies have demonstrated that FSH, LH, and estradiol stimulate ovarian epithelial cell proliferation and inhibit apoptosis in ovarian epithelial cancer cell lines[81], while it seems that approximately half of all ovarian epithelial tumors express gonadotropin receptors[82]. Nevertheless, the majority of studies, evaluating the risk of invasive ovarian cancer following the use of fertility drugs when compared with infertile controls and/or with the general population have shown no significant increase. The largest systematic review was performed by the Cochrane Collaboration Group, including a total of 182,972 women with exposure to ovarian stimulating drugs for treatment of subfertility and histologically confirmed borderline or invasive ovarian cancer[83]. Overall, the collaboration group concluded that there was no convincing evidence that fertility drugs were associated with an increased risk of invasive ovarian cancer. On the contrary, several studies have shown a small increase in the absolute risk of borderline ovarian tumors after fertility treatments but it is important to note that these tumors are indolent, demonstrate low malignant potential and generally have a favorable prognosis. Thus, current guidance concludes that there is insufficient evidence to recommend against the use of fertility medications to avoid borderline ovarian tumors.

Current scientific data concerning the pathogenesis of breast cancer suggest that it is associated to a hormonal etiology and consequently ovulation induction medications could contribute to cancer development. In general, exposure to endogenous estrogen increases the risk (earlier menarche, delayed menopause) and despite the fact that progesterone appears protective to the endometrium, it is mitogenic to the breast[84]. The majority of studies and systematic reviews have failed to show an association between fertility drugs and breast cancer, although there are some that demonstrate conflicting data, regarding the cumulative dose of the drugs or the onset of treatment at a young age[85].

Regarding type 1 endometrial cancer, which is the most common, appears to be multifactorial. More specifically, it is associated with unopposed estrogen activity, anovulation, progesterone deficiency and obesity. When evaluating the relationship between fertility drug use and subsequent development of endometrial cancer, the majority of studies showed that the overall use of fertility drugs, specifically CC and gonadotropins was not associated with a significantly increased risk[76, 86, 87]. However, infertility appeared to be an important independent risk factor and uterine cancer incidence among women of this category, was notably increased.

Along with other gynecological cancers, several studies evaluated the risk of cervical cancer following the use of ovulation induction drugs and found no increased risk when compared to the general population as well as patients with infertility[36].

A large retrospective cohort study, examined the effects of fertility drugs on non gynecological cancers, such as thyroid cancer, colorectal cancer and melanoma[88]. Authors conclude that there is an increased risk of melanoma and a non statistically significant increased risk of thyroid cancer, while colon cancer was not associated at all.

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Based on the current literature, patients should be counseled that infertile women may face an increased risk of invasive ovarian, endometrial and breast cancer, however, use of fertility drugs does not appear to increase this risk. Moreover, international health organizations, such as NICE (2013) recommend that ovarian stimulation agents should be used to the minimum effective dose and duration of use. Health providers should inform women that while the absolute risks of long-term adverse outcomes of IVF treatment, with or without ICSI are low, a small increased risk of borderline ovarian tumors can not be excluded.

Conclusions

Assisted reproduction is a continuously evolving dynamic procedure. Based on the fact that science is targeted applying tailored treatments for each patient, IVF could not lack behind. The ability to have one protocol for each infertile patient seems ideal and needs more research and breakthrough discoveries. The existing protocols are always adjusted to the needs of each woman while poor responders and PCOS patients are the ultimate challenges of the future.

Moreover, patients who undergo treatment for gynecologic malignancies are another category, in which we need to focus on. In this group, effective and of short duration protocols are of outmost importance, as it is understood, that in this cases, time matters the most. However, the safety of the patients must always be our first priority and research on the safety of ovulation induction drugs and their relationship with carcinogenesis is yet to be established.

References

1. Fauser BC, Van Heusden AM. Manipulation of human ovarian function: physiological concepts and clinical consequences. Endocr Rev 1997; 18(1):71-106.

2. Gougeon A. Regulation of ovarian follicular development in primates: facts and hypotheses. Endocr Rev 1996;17(2): 121-55.

3. McGee EA, Hsueh AJ. Initial and cyclic recruitment of ovarian follicles. Endocr Rev 2000; 21(2):200-14.

4. BakerTG. A Quantitative and Cytological Study of Germ Cells in Human Ovaries. Proc R Soc Lond B Biol Sci 1963;158:417-33.

5. Brown JB. Pituitary control of ovarian function--concepts derived from gonadotrophin therapy. Aust N Z J Obstet Gynaecol 1978;18(1):46-54.

6. Baird DT. A model for follicular selection and ovulation: lessons from superovulation. J Steroid Biochem 1987;27(1-3):15-23.

7. Fauser BC, Donderwinkel P, Schoot DC. The step-down principle in gonadotrophin treatment and the role of GnRH analogues. Baillieres Clin Obstet Gynaecol 1993;7(2): 309-30.

8. Karsch FJ et al. An analysis of the negative feedback control of gonadotropin secretion utilizing chronic implantation of ovarian steroids in ovariectomized rhesus monkeys. Endocrinology 1973; 93(2): 478-86.

9. Roseff SJ, Bangah ML, Kettel LM et al. Dynamic changes in circulating inhibin levels during the luteal-follicular transition of the human menstrual cycle. J Clin Endocrinol Metab 1989;69 (5):1033-9.

10. Zeleznik AJ, Schuler HM, Reichert LE Jr. Gonadotropin-binding sites in the rhesus monkey ovary: role of the vasculature in the selective distribution of human chorionic gonadotropin to the preovulatory follicle. Endocrinology 1981; 109(2): 356-62.

11. Goff AK, Armstrong DT. Stimulatory action of gonadotropins and prostaglandins on adenosine-3',5'-monophosphate production by isolated rat granulosa cells. Endocrinology 1977;101(5): 1461-7.

12. Richards JS, Russell DL, Robker RL, Dajee M, Alliston TN. Molecular mechanisms of ovulation and luteinization. Mol Cell Endocrinol 1998;145(1-2): 47-54.

13. Boostanfar R, Jain JK, Mishell DR Jr, Paulson RJ. A prospective randomized trial comparing clomiphene citrate with tamoxifen citrate for ovulation induction. Fertil Steril 2001;75(5): 1024-6.

14. Fisher SA, Reid RL, Van Vugt DA, Casper RF. A randomized double-blind comparison of the effects of clomiphene citrate and the aromatase inhibitor letrozole on ovulatory function in normal women. Fertil Steril 2002;78(2):280-5.

15. Kettel LM, Roseff SJ, Berga SL, Mortola JF, Yen SS. Hypothalamic-pituitary-ovarian response to clomiphene citrate in women with polycystic ovary syndrome. Fertil Steril 1993; 59(3):532-8.

16. Filicori M, Cognigni GE, Samara A et al. The use of LH activity to drive folliculogenesis: exploring uncharted territories in ovulation induction. Hum Reprod Update 2002; 8(6):543-557.

17. Filicori M, Cognigni GE, Tabarelli C et al. Stimulation and growth of antral ovarian follicles by selective LH activity administration in women. J Clin Endocrinol Metab 2002; 87(3): 1156-1161.

18. Messinis IE. Ovulation induction: a mini review. Human Reproduction 2005;20(10): 2688-2697.

19. Al-Inany HG, Youssef MA, Aboulghar M et al. Gonadotrophin-releasing hormone antagonists for assisted reproductive technology. Cochrane Database Syst Rev 2016; 4: Cd001750.

20. Homburg R. Clomiphene citrate--end of an era? A mini-review. Hum Reprod 2005; 20(8):2043-51.

21. Kousta E, White DM, Franks S. Modern use of clomiphene citrate in induction of ovulation. Hum Reprod Update 1997; 3(4): 359-65.

22. Misso ML, Wong JL, Teede HJ et al. Aromatase inhibitors for PCOS: a systematic review and meta-analysis. Hum Reprod Update 2012; 18(3): 301-12.

23. Committee Opinion No. 663: Aromatase Inhibitors in Gynecologic Practice. Obstet Gynecol 2016;127(6):e170-4.

24. Hoffman BL et al. Treatment of the Infertile Couple. Williams Gynecology, McGraw-Hill Education: 2016,United States of America :449-470.

25. Lin H, Li Y, Li L, Wang W, Yang D, Zhang Q . Is a GnRH Antagonist Protocol Better in PCOS Patients? A Meta-Analysis of RCTs. PLoS ONE 2014;9(3):e91796.

26. Morgante G, Cappelli V, Di Sabatino A, Massaro MG, De Leo V. Polycystic ovary syndrome (PCOS) and hyperandrogenism: the role of a new natural association. Minerva Ginecol 2015; 67(5): 457-63.

27. Thessaloniki ESHRE/ASRM-Sponsored PCOS Consensus Workshop Group. Consensus on infertility treatment related to polycystic ovary syndrome. Hum Reprod 2008; 23(3): 462-77.

28. Practice Committee of American Society for Reproductive Medicine. Definitions of infertility and recurrent pregnancy loss: a committee opinion. Fertility and Sterility. 99(1):63.

29. Melo AS, Ferriani RA, Navarro PA. Treatment of infertility in women with polycystic ovary syndrome: approach to clinical practice. Clinics (Sao Paulo) 2015; 70(11):765-9.

30. Jayagopal V, Kilpatrick ES, Holding S, Jennings PE, Atkin SL. Orlistat is as beneficial as metformin in the treatment of polycystic ovarian syndrome. J Clin Endocrinol Metab 2005; 90(2):729-33.

31. Moran LJ, Brinkworth G, Noakes M, Norman RJ. Effects of lifestyle modification in polycystic ovarian syndrome. Reprod Biomed Online 2006;12(5):569-78.

32. Moran LJ, Noakes M, Clifton PM, Tomlinson L, Galletly C, Norman RJ. Dietary composition in restoring reproductive and metabolic physiol-

ogy in overweight women with polycystic ovary syndrome. J Clin Endocrinol Metab 2003; 88(2): 812-9.

33. Stamets K, Taylor DS, Kunselman A, Demers LM, Pelkman CL, Legro RS. A randomized trial of the effects of two types of short-term hypocaloric diets on weight loss in women with polycystic ovary syndrome. Fertil Steril 2004; 81(3):630-7.

34. Tang T, Glanville J, Hayden CJ, White D, Barth JH, Balen AH. Combined lifestyle modification and metformin in obese patients with polycystic ovary syndrome. A randomized, placebo-controlled, double-blind multicentre study. Hum Reprod 2006; 21(1):80-9.

35. Imani B, Eijkemans MJ, te Velde ER, Habbema JD, Fauser BC. A nomogram to predict the probability of live birth after clomiphene citrate induction of ovulation in normogonadotropic oligoamenorrheic infertility. Fertil Steril 2002; 77(1):91-7.

36. Perales-Puchalt A and Legro RS, Ovulation induction in women with polycystic ovary syndrome. Steroids 2013; 78(8):767-72.

37. Imani B, Eijkemans MJ, te Velde ER, Habbema JD, Fauser BC.Predictors of chances to conceive in ovulatory patients during clomiphene citrate induction of ovulation in normogonadotropic oligoamenorrheic infertility. J Clin Endocrinol Metab 1999; 84(5):1617-22.

38. Lambalk CB, Huirne JAF, Macklon NS et al.Ovulation induction with clomiphene or gonadotropins. International Congress Series, 2005. 1279:16-23.

39. Saha L, Kaur S, and Saha PK. N-acetyl cysteine in clomiphene citrate resistant polycystic ovary syndrome: A review of reported outcomes. Journal of Pharmacology & Pharmacotherapeutics, 2013; 4(3):187-191.

40. Overbeek A, Kuijper EAM et al. Clomiphene citrate resistance in relation to follicle-stimulating hormone receptor Ser680Ser-polymorphism in polycystic ovary syndrome. Hum Reprod 2009; 24(8):2007-13.

41. Kolibianakis EM, Zikopoulos KA, Fatemi HM et al. Endometrial thickness cannot predict ongoing pregnancy achievement in cycles stimulated with clomiphene citrate for intrauterine insemination. Reprod Biomed Online 2004; 8(1):115-8.

42. Nahuis MJ, Oosterhuis GJ, Hompes PG et al. The basic fertility workup in women with polycystic ovary syndrome: a systematic review. Fertility and Sterility 2013; 100(1): 219-225.

43. Balen AH. Ovulation induction in the management of anovulatory polycystic ovary syndrome. Mol Cell Endocrinol 2013; 373(1-2):77-82.

44. Balasch J, Fabregues F, Creus M et al. Follicular development and hormone concentrations following recombinant FSH administration for anovulation associated with polycystic ovarian syndrome: prospective, randomized comparison between low-dose step-up and modified step-down regimens. Human Reproduction 2001; 16(4): 652-656.

45. Christin-Maitre S and Hugues JN. A comparative randomized multicentric study comparing the step-up versus step-down protocol in polycystic ovary syndrome. Human Reproduction 2003;18 (8):1626-1631.

46. Van Santbrink EJ, Donderwinkel PF, Van Dessel TJ et al. Gonadotrophin induction of ovulation using a step-down dose regimen: single-centre clinical experience in 82 patients. Hum Reprod 1995;10(5):1048-53.

47. Homburg R, Howles CM. Low-dose FSH therapy for anovulatory infertility associated with polycystic ovary syndrome: rationale, results, reflections and refinements. Hum Reprod Update 1999; 5(5):493-9.

48. Balasch J, Tur R, Alvarez P et al. The safety and effectiveness of stepwise and low-dose adminis-

tration of follicle stimulating hormone in WHO group II anovulatory infertile women: evidence from a large multicenter study in Spain. J Assist Reprod Genet 1996; 13(7):551-6.

49. Eijkemans MJ, Imani B, Mulders AG, Habbema JD, Fauser BC. High singleton live birth rate following classical ovulation induction in normogonadotrophic anovulatory infertility (WHO 2). Hum Reprod 2003;18(11):2357-62.

50. Flyckt RL, Goldberg JM. Laparoscopic ovarian drilling for clomiphene-resistant polycystic ovary syndrome. Semin Reprod Med 2011;29(2): 138-46.

51. Mitra S, Nayak PK, Agrawal S. Laparoscopic ovarian drilling: An alternative but not the ultimate in the management of polycystic ovary syndrome. J of Nat Sci Biol and Med 2015;6(1): 40-48.

52. Seow KM, Juan CC, Hwang JL, Ho LT. Laparoscopic surgery in polycystic ovary syndrome: reproductive and metabolic effects. Semin Reprod Med 2008; 26(1):101-10.

53. Melica F, Chiodi S, Cristoforoni PM, Ravera GB. Reductive surgery and ovarian function in the human--can reductive ovarian surgery in reproductive age negatively influence fertility and age at onset of menopause? Int J Fertil Menopausal Stud 1995;40(2): 79-85.

54. Gynaecologists, R.C.o.O.a., Polycystic Ovary Syndrome, Long-term Consequences (Green-top Guideline No. 33). 2014.

55. Vause TD, Cheung AP, Sierra S et al. Ovulation induction in polycystic ovary syndrome: No. 242, May 2010. Int J Gynaecol Obstet 2010;111(1): 95-100.

56. ACOG Practice Bulletin No. 108: Polycystic ovary syndrome. Obstet Gynecol 2009; 114(4):936-49.

57. Farquhar C, Brown J, Marjoribanks J. Laparoscopic drilling by diathermy or laser for ovulation induction in anovulatory polycystic ovary syndrome. Cochrane Database of Systematic Reviews2012;(6).

58. Unlu C, Atabekoglu CS. Surgical treatment in polycystic ovary syndrome. Current Opinion in Obstetrics and Gynecology 2006;18(3):286-292.

59. Amer SA, Li TC, Metwally M, Emarh M, Ledger WL. Randomized controlled trial comparing laparoscopic ovarian diathermy with clomiphene citrate as a first-line method of ovulation induction in women with polycystic ovary syndrome. Hum Reprod 2009; 24(1): 219-25.

60. Abu Hashim H1, Al-Inany H, De Vos M, Tournaye H. Three decades after Gjonnaess's laparoscopic ovarian drilling for treatment of PCOS; what do we know? An evidence-based approach. Arch Gynecol Obstet 2013;288(2):409-22.

61. Xiao J, Chen S, Zhang C, Chang S. Effectiveness of GnRH antagonist in the treatment of patients with polycystic ovary syndrome undergoing IVF: a systematic review and meta analysis. Gynecological Endocrinology 2013; 29(3):187-191.

62. Heijnen EM, Eijkemans MJ, Hughes EG et al. A meta-analysis of outcomes of conventional IVF in women with polycystic ovary syndrome. Hum Reprod Update 2006. 12(1):13-21.

63. Tang T, Lord JM, Norman RJ, Yasmin E, Balen AH. Insulin-sensitising drugs (metformin, rosiglitazone, pioglitazone, D-chiro-inositol) for women with polycystic ovary syndrome, oligo amenorrhoea and subfertility. Cochrane Database Syst Rev 2012(5): Cd003053.

64. Palomba S, Falbo A, La Sala GB. Metformin and gonadotropins for ovulation induction in patients with polycystic ovary syndrome: a systematic review with meta-analysis of randomized controlled trials. Reproductive Biology and Endocrinology : RB&E 2014; 12:3-3.

65. Palomba S, Falbo A, La Sala GB. Effects of metformin in women with polycystic ovary syndrome treated with gonadotrophins for in vitro fertilisation and intracytoplasmic sperm injec-

cancer in women treated with ovarian stimulating drugs for infertility. Cochrane Database Syst Rev 2013; (8):CD008215.

84. Yager JD, Davidson NE. Estrogen carcinogenesis in breast cancer. N Engl J Med 2006; 354(3):270-82.

85. Stewart LM, Holman CD, Hart R, Bulsara MK, Preen DB, Finn JC. In vitro fertilization and breast cancer: is there cause for concern? Fertil Steril 2012: 98(2):334-40.

86. Althuis MD, Moghissi KS, Westhoff CLet al. Uterine cancer after use of clomiphene citrate to induce ovulation. Am J Epidemiol 2005;161(7): 607-15.

87. Lerner-Geva L, Rabinovici J, Olmer L, Blumstein T, Mashiach S, Lunenfeld B. Are infertility treatments a potential risk factor for cancer development? Perspective of 30 years of follow-up. Gynecol Endocrinol 2012; 28(10): 809-14.

88. Hannibal CG, Jensen A, Sharif H et al. Malignant melanoma risk after exposure to fertility drugs: results from a large Danish cohort study. Cancer Causes Control 2008; 19(7):759-65.