European Review for Medical and Pharmacological Sciences 2013; 17: 853-873

GnRH antagonists in assisted reproductive techniques: a review on the Italian experience

R. MARCI[1], A. GRAZIANO[1], G. LO MONTE[1], I. PIVA[1], I. SOAVE[1], E. MARRA[1], F. LISI[2], M. MOSCARINI[3], D. CASERTA[3]

[1]Department of Morphology, Surgery and Experimental Medicine, Section of Obstetrics and Gynecology, University of Ferrara, Ferrara, Italy.
[2]CERMER, Centro Ricerca Medicina della Riproduzione, Clinica Villa Mafalda, Rome, Italy
[3]Department of Woman Health and Territory's Medicine, University of Rome Sapienza, S. Andrea Hospital, Rome, Italy

Abstract. – Current Controlled Ovarian Stimulation (COH) for Assisted Reproductive Techniques (ART) pursues three main objectives: hypophyseal activity suppression, multiple follicle growth stimulation, and ovulation induction. By suppressing hypophyseal activity, it is possible to prevent untimely LH surge and allow the appropriate development of the leading follicle. The classical GnRH agonist long protocol is the most widely used in COH for ART. However, an alternative regimen based on GnRH antagonist has been recently introduced in clinical practice. As competitive antagonists, these drugs display an immediate and quickly reversible effect and they avoid hormonal withdrawal side effects. Moreover, this protocol shows undeniable advantages, including the shorter duration of the treatment, the lower amount of gonadotropin required, the shorter hormonal and ultrasound monitoring of patients, milder physical and emotional stress, and a lower risk of Ovarian Hyperstimulation Syndrome (OHSS). The use of GnRH antagonists was traditionally restricted to selected patients, as "poor responders" and women at high-risk of developing OHSS such as Polycystic Ovary Syndrome (PCOS) and patients who had previously experienced OHSS. These findings could prompt a trend to change from the standard agonist protocol to the antagonist protocol in all categories of patients. This review provides a comprehensive overview of the use of GnRH antagonist protocols applied both to IVF techniques and to IUI procedures in the Italian experience.

Key Words:
GnRH Antagonist, Assisted reproductive techniques, In vitro fertilization, Controlled ovarian stimulation, Cetrorelix, Intrauterine insemination.

Introduction

Controlled ovarian hyperstimulation (COH) plays a meaningful role in reproductive medicine and is of major importance in achieving a pregnancy through assisted reproductive technology (ART). COH grants higher pregnancy and implantation rates than natural cycles[1-2]. Current COH for ART pursues three main objectives: hypophyseal activity suppression, multiple follicle growth stimulation, and ovulation induction[3].

In fact, during stimulated cycles multifollicular recruitment determines a rapid increase in estradiol (E_2) levels, resulting in an untimely luteinizing hormone (LH) release. Premature LH surge occurs in 24% of IUI cycles and it is held to be responsible for the luteinisation and disruption of normal follicle and oocyte development[4], thus possibly leading to cycle cancellation[5-7].

By suppressing hypophyseal activity, it is possible to prevent untimely LH surge and allow the appropriate development of the leading follicle. Two kinds of drugs are currently available for this purpose: GnRH agonists (GnRH-a) and GnRH antagonists (GnRH-ant). For years GnRH-a have been the most widely used in ART, while GnRH-ant were introduced in clinical practice only in the last decades, as a valid alternative to the classical protocol[5,7-16]. Although these drugs share common clinical indications, their mechanisms of action are completely different[17].

GnRH-a determine internalization of the ligand/receptor complex and decrease the number of receptors (downregulation)[4]. Thus, the pituitary gland becomes refractory to the stimulatory effect of endogenous GnRH, gonadotropin pulsatility is significantly reduced and sex steroid concentrations approach or equal values observed following surgical castration. Contrary to GnRH-a, GnRH antagonists are competitive inhibitors of GnRH binding to its receptors[4,11-12]. Therefore they induce a direct, dose-dependent and quickly reversible block of GnRH-receptors,

Corresponding Author: Roberto Marci, Ph.D, MD; e-mail: roberto.marci@unife.it

avoiding a flare effect. Acute administration of GnRH-ant suppresses gonadotropin and sex-steroid secretion rapidly and dose-dependently in animals and in humans[4,11-12].

On the grounds of the currently available evidence, we aim at reviewing Italian studies dealing with the clinical use of GnRH-ant in ovarian stimulation protocols either applied to *in vitro* fertilization (IVF) techniques or to intra-uterine insemination (IUI) procedures. Furthermore, the effect of these drugs on follicular environment will be evaluated.

GnRH-Antagonists in vitro Fertilization (IVF)-intracytoplasmic Sperm Injection (ICSI)

GnRH-ant are administered in the late follicular phase according to three possible protocols: the single-dose fixed protocol, the multiple-dose fixed protocol and the multiple-dose flexible protocol (see Figure 1 for GnRH-ant protocols and Figure 2 for GnRH-a protocols). In the single-dose fixed protocol a single GnRH-ant injection is given on day 7-8. Alternatively 0.25 mg GnRH-ant can be administered from day 5-7 un-

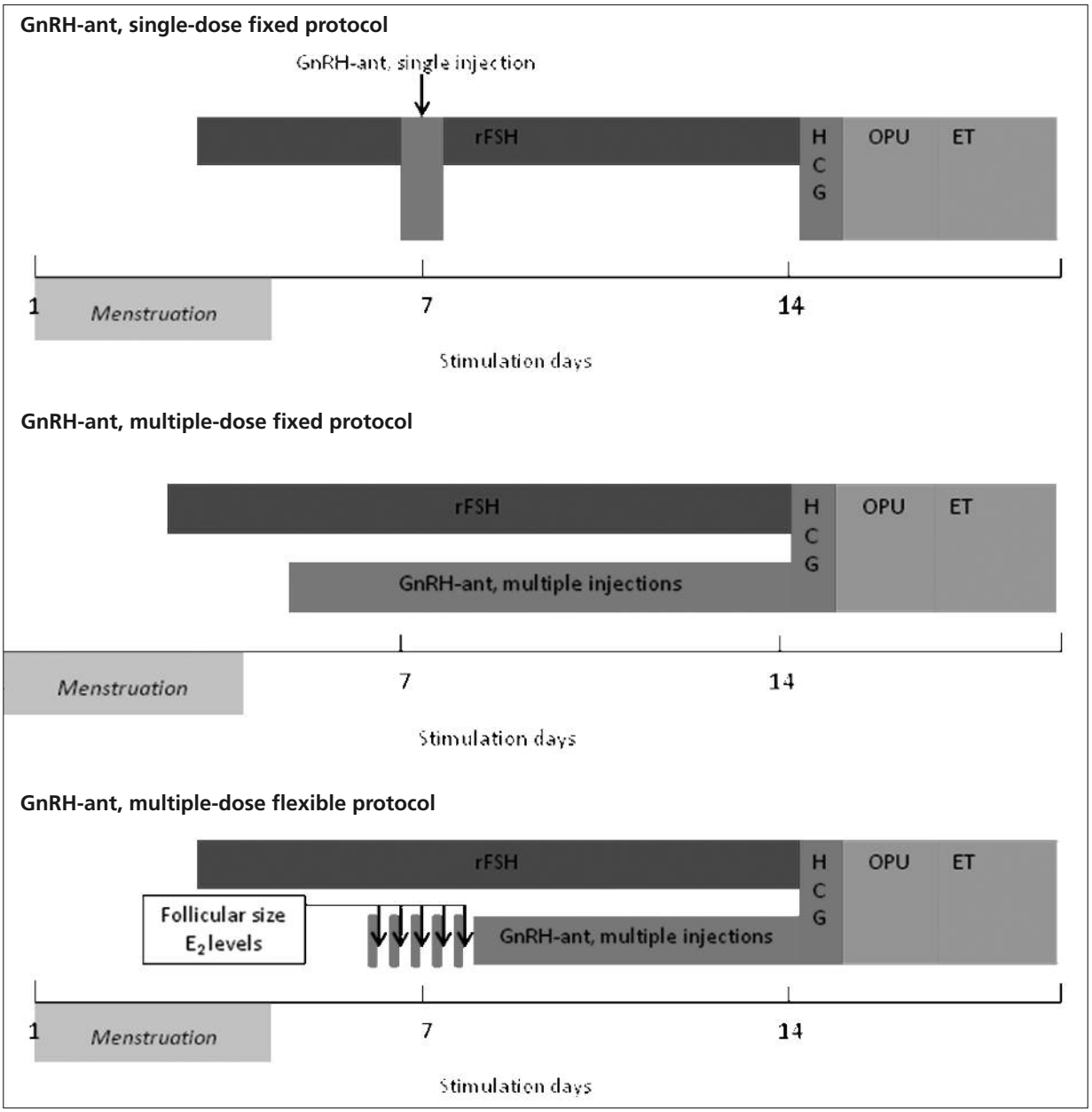

Figure 1. GnRH-antagonists stimulation protocols.

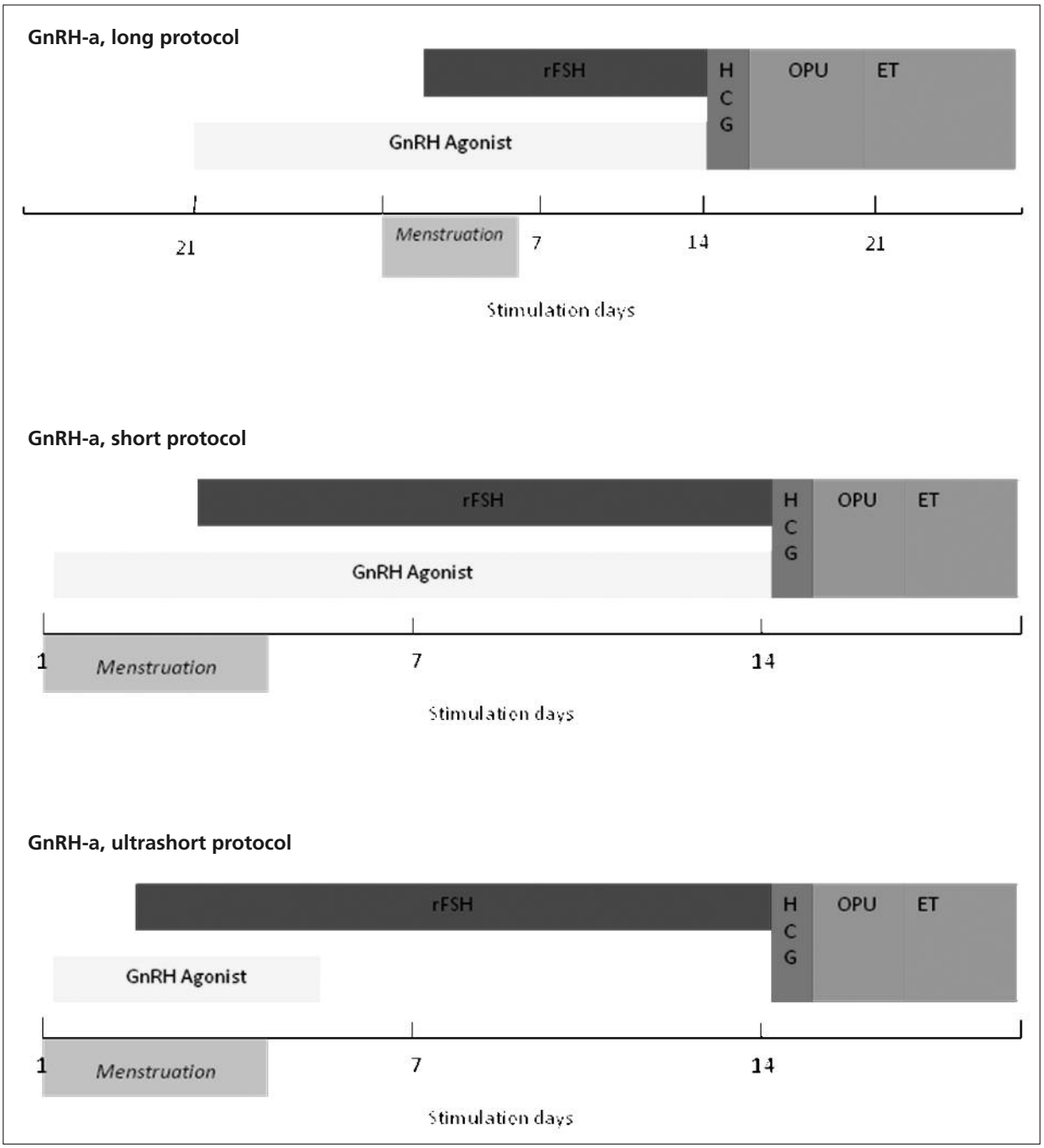

Figure 2. GnRH-agonists stimulation protocols

til the day of human chorionic gonadotropin (hCG) administration, according to a multiple-dose fixed protocol. This approach effectively prevents premature LH surge and it is associated with good clinical outcomes[8-9]. The multiple-dose protocol and the single-dose protocol are equally effective in the prevention of premature LH surge over a wide range of body weights[18]. In the multiple-dose flexible protocol GnRH-ant administration is not started on a fixed day but it is timed according to the follicular size and to E_2 levels. The multiple-dose flexible protocol seems as effective as the multiple-dose fixed protocol, but it is associated with higher E_2 levels and reduces the total amount of GnRH-ant used[19].

Many randomized controlled trials (RCTs) have compared the GnRH-a long luteal protocol to the GnRH-ant protocol both in single-[20] and in

multiple-dose [9]. The GnRH-ant based protocols allowed to reduce the dose of gonadotropins used as well as the duration of the stimulation regimens. Besides, the GnRH-ant granted a more physiological pattern of follicular recruitment, with fewer small antral follicles and lower E_2 levels, reducing the risk of severe ovarian hyperstimulation syndrome (OHSS). However, the oocytes retrieved were significantly fewer and a trend towards lower pregnancy rate (PR) could be noticed in most of the RCTs. Five of these RCTs were examined in a meta-analysis published in a Cochrane review, that confirmed the previous findings as regards the duration of the stimulation protocol, the amount of gonadotropins used, the number of oocytes retrieved and the E_2 levels on the day of hCG administration. Additionally, this meta-analyses showed a significant reduction in clinical pregnancy rate and failed to prove a significant preventive effect over severe OHSS[11]. The initial reservations about GnRH-ant protocols led to their use as a second choice, often in cycles with unfavorable *a priori* prognosis[21].

However, a 5% higher clinical pregnancy rate did not match with an increase in live-birth rate according to a subsequent meta-analysis comparing GnRH-a and GnRH-ant[22]. Moreover, a similar PR was confirmed by the subanalysis of patients with equal demographic and clinical features who were administered either a GnRH-ant or a GnRH-a[23]. In addition to that, the greater safety of GnRH-ant over GnRH-a has been definitively demonstrated in the last version of the Cochrane review[12], in which a further clinical advantage has been detected, i.e. the reduction in the number of cycles cancelled due to OHSS risk. Furthermore, this review suggests that GnRH-ant administration provides comparable results to traditional GnRH-a stimulation, as opposed to previous works. In particular no significant differences concerning live-birth rates, ongoing pregnancy rates, miscarriage rates per clinical pregnancy rate, and rates of cancellation due to poor ovarian response were reported.

Besides preventing OHSS, GnRH-ant reduce the duration of ovarian stimulation, resulting in a shorter hormonal and ultrasonographic monitoring of patients[24] and avoiding the hypoestrogenic side effects (e.g. hot flushes, sleep disturbances, headaches), which are frequently observed with GnRH agonists[12,25]. Despite the undeniable advantages provided by GnRH-ant, their efficacy is still debated. Therefore, scientific research on the use of GnRH-ant in COH for IVF/ICSI has been promoted even in recent years. As to the Italian experience, the efficacy and safety of GnRH-ant has been tested both in normal-prognosis patients and in specific categories, in which the administration of such drugs could be beneficial. The application of GnRH-ant to selected subsets of women is presented separately in the next sections.

GnRH Antagonists in Normal Responder Patients

Although GnRH antagonists have been used for more than 10 years, there is still no general agreement on their efficacy in COH for ART cycles. As shown by literature, the GnRH antagonists are as effective as the GnRH-agonists long protocol in younger patients (< 35 years old) and in women with normal ovarian response, with the advantages of reducing the dose of FSH needed to stimulate the ovary, and of decreasing the risk of OHSS[26].

Several recent Italian studies addressed the topic, comparing the GnRH-a long protocol and the GnRH-ant regimen in normal responders (Table I). De Palo et al[27] work included 136 consecutive patients undergoing COH/ICSI. The women enrolled were randomly assigned to one of two possible protocols: the GnRH-a long protocol or the GnRH-ant protocol. The total amount of rFSH used, the stimulation length, the LH level and the number of follicles sized ≥ 18 mm did not differ significantly between the groups. Besides, the implantation rate, the clinical PR and the miscarriage rate were similar in the two groups. However GnRH-a protocol was associated with higher E_2 levels and with a higher number of follicles on the day of hCG administration. Moreover, this regimen was related to an increased number of cycles with egg retrieval. As explained by the Authors, the results of the study can be attributed to the better synchronization of follicular development allowed by GnRH-a that increased the total number of oocytes retrieved. Alternatively, the reduced follicular recruitment in the GnRH-ant group could be due to an elevated LH activity before Cetrorelix administration. On the one hand, high LH levels can induce androgen-mediated atresia of small follicles; on the other hand they can support the growth of the larger follicles, whose granulose cells have begun to express LH receptors. In conclusion, both regimens showed a similar efficacy in terms of implantation and pregnancy rates, but

Table I. Italian studies on the use of GnRH antagonists in controlled ovarian hyperstimulation for *in vitro* fertilization in normal responders.

Publication	Population (n/n)	GnRH-ant protocol	Other protocol	Trigger for ovulation	Main results
Depalo R et al (2009)	67/69	Multiple-dose	Long GnRH-a flexible protocol	hCG (6500 IU) when two or more follicles > 17 mm	No. of oocytes retrieved: GnRH-a > GnRH-ant, $p < 0.02$ No. of mature oocytes: GnRH-a > GnRH-ant, $p < 0.01$ Quality of embryos, implantation rate, Clinical PR, Ongoing PR, Miscarriage rate: NS
Greco E et al (2007)	200/200	Multiple-dose fixed protocol	Long GnRH-a protocol	hCG (10,000 IU) when at least 3 follicle ≥ 17 mm	Total r-FSH dose: GnRH-a > GnRH-ant, $p < 0.01$ Duration of stimulation, no. of oocytes and no. of MII oocytes retrieved: GnRH-a > GnRH-ant, $p < 0.05$ Quality of embryos, Implantation rate, Clinical PR, Overall PR: NS
Casano S et al (2012)	205/207	Mild GnRH-ant protocol	Long GnRH-a protocol	hCG (10,000 IU) when at least 2 follicle ≥ 18 mm	Total r-FSH dose: GnRH-a > GnRH-ant, $p < 0.001$ No. of oocytes retrieved, Quality of embryos, Implantation rate, Clinical PR, Cumulative PR: NS
Marci R et al (2013)	91/156	Multiple-dose flexible protocol	Long GnRH-a protocol	hCG (6500 IU) r- when at least 2 follicle ≥ 17 mm	Total r-FSH dose: GnRH-a > GnRH-ant, $p = 0.0014$ Duration of stimulation: GnRH-a > GnRH-ant, $p < 0.001$ No. of oocytes and no. of MII oocytes retrieved, Implantation rate, Clinical PR, Ongoing PR: NS
Levi-Setti PE et al (2006)	20/20	Multiple-dose flexible protocol with r-FSH alone	Multiple-dose flexible protocol with r-FSH/r-LH	hCG (10,000 IU) when at least 3 follicles > 18 mm	E2 (hCG day): r-LH/GnRH-ant > GnRH-ant, $p < 0.01$ No. of oocytes and no. of MII oocytes retrieved, Implantation rate, PR: NS

hCG: human chorionic gonadotropin; PR: pregnancy rate; NS: not significant; MII: metaphase II; E_2: estradiol.

the administration of GnRH-a allowed a better follicular growth and oocyte development[27]. Accordingly, an older study by Greco et al[24] showed a comparable effectiveness of the two drugs in terms of implantation rate and PR, but reported a lower amount of rFSH administered and a reduced length of stimulation when GnRH-ant were used. Additionally, the GnRH-ant protocol was associated with an increase in patients' satisfaction[24]. It can be inferred that the GnRH-ant protocol grants comparable results to the GnRH-a long protocol in young women with normal

ovarian reserve, and it is more patient-friendly. Since this regimen is particularly attractive in terms of social and medical costs, every effort should be made to uncover why PR are slightly reduced in the GnRH-ant cycles. In the Authors' opinion, the main factors reducing PR in GnRH-ant protocol are the quantitative and qualitative characteristics of the oocytes retrieved. In fact it has been suggested that GnRH-ant may increase the number of follicles arrested in the primordial stage[8,24]. These drugs may interfere negatively with follicular granulose cell division and cell signaling pathway driving the oocyte into meiosis II [24]. However, higher PR can be achieved through (1) cycle delay or cancellation in patients with initially elevated E_2 and P concentrations[28]; (2) early administration of hCG limiting luteal phase duration[29]; (3) prolongation of luteal phase support[28]; (4) active oocyte selection[24]. Recently, two subsequent studies[30-31] evaluating the efficacy of GnRH-ant when compared to GnRH-a, showed the same results: the use of Cetrorelix resulted in a significant reduction in both the duration and the amount of gonadotropins used, and in similar pregnancy outcomes.

Many studies suggest that the suppression of LH caused by GnRH-ant might have a detrimental effect on IVF outcomes. In 2006 Levi-Setti et al[32] evaluated the addition of r-LH to the traditional r-FSH/GnRH-ant protocol. The use of r-LH in combination with r-FSH prevented a decrease in estradiol after GnRH antagonist administration, but it did not influence IVF outcomes.

In synthesis, the Italian studies dealing with the administration of GnRH-ant to normal responders have demonstrated an efficacy overall comparable to GnRH-a. Yet the PR provided by GnRH-ant are slightly lower, probably because of the negative interference with follicular growth and oocyte development. Many procedures may be adopted to increase the PR associated with the GnRH-ant, as proposed by Greco. In fact, every effort should be made to introduce the GnRH-ant in clinical practice because this protocol is more patient-friendly and almost as effective as the traditional long protocol.

GnRH Antagonists in Poor Responder Patients

The definition of "poor response" (POR) to COH for IVF or ICSI is heterogeneous and corresponds either to one parameter or to a combination of more, as the number of mature follicles observed on ultrasound scans (ranging from < 2 to < 5), the peak of serum E_2 levels during COH (ranging from 100 to 600 pg/Ml), the number of mature oocytes retrieved (< 3 to < 6), the minimal cumulative dose or the days of GT stimulation required in a prior cycle. Furthermore, age (> 40 years), early follicular phase FSH levels (ranging from 6.5 to 15 mIU/mL) and failed GnRH-a stimulation test are known to be associated with poor response[33]. However, the most acceptable definition is based upon the number of mature ovarian follicles under ultrasound (generally < 3 follicles measuring ≥ 17 mm in diameter) and consequently the number of retrieved oocytes (< 3)[33]. A diagnosis of POR is then confirmed by the failure of a standard (long protocol) ovarian stimulation or by the cancellation of at least one IVF cycle[34]. In the light of the high prevalence of POR among patients undergoing IVF (9-24%)[33-34], appropriate criteria for its diagnosis were needed. According to ESHRE, the diagnosis of POR in IVF is based on at least two of the following three features: (1) advanced maternal age or any other risk factor for POR; (2) a previous POR; and (3) an abnormal ovarian reserve test (ORT). Advanced age patients presenting an abnormal ORT may be classified as "expected poor responder" since both of these parameters may indicate reduced ovarian reserve[35]. As to the therapy, there is presently insufficient evidence to support the routine use of any particular intervention either for pituitary down regulation, or ovarian stimulation or adjuvant therapy in the management of poor responders to COH in IVF. However, many protocols have been proposed to increase ovarian response[33-34]. GnRH-a regimen represents one of the major approaches used in poor responders in order to improve ART outcome. However, in these regimens LH levels rise during the early follicular maturation due to the flare-up effect, resulting in an impaired oocyte quality[36]. On the contrary, GnRH-ant administration causes a rapid and profound suppression of pituitary function and hence it is not associated with an increased endogenous gonadotropin release. Furthermore, GnRH-ant administration is restricted to the mid-late follicular phase so it prevents premature LH surge without causing suppression in the early follicular phase. Thus the GnRH-ant regimen was considered a suitable protocol for ovulation induction in poor responders. Yet the results are conflicting probably because of the different criteria of patients selection, the lack of uniformity in the definition of "poor responders"[34-37], the different protocols used for ovarian stimulation, and the small number of women in-

cluded in each study[26] (Table II). Some Authors suggest that GnRH-ant administration expands the pool of retrieved oocytes, without impairing ART outcome[38], other studies show that GnRH-a administration, especially the flare-up protocol[37], is more advantageous than GnRH-ant in poor responders. As to the studies supporting the effectiveness of GnRH-ant administration in poor responders, Marci's work showed that Cetrorelix protocol could be considered a good alternative to

Table II. Italian studies on the use of GnRH antagonists in controlled ovarian hyperstimulation for *in vitro* fertilization in poor responders.

Publication	Population (n/n)	GnRH-ant protocol	Other protocol	Trigger for ovulation	Main results
Marci R et al (2005)	30/30	Multiple-dose flexible protocol	Long GnRH-a protocol	hCG (10,000 IU) when at least 2 follicles ≥ 17 mm	Duration of stimulation: GnRH-a > GnRH-ant, $p < 0.01$ Total r-FSH dose: GnRH-a > GnRH-ant, $p < 0.01$ No. of oocytes retrieved: GnRH-ant > GnRH-a, $p < 0.02$ No. of follicles > 15 mm (hCG day): GnRH-ant > GnRH-a, $p < 0.01$ Endometrial thickness, Clinical PR per embryo transfer: NS
Malmusi S et al (2005)	25/30	Multiple-dose flexible protocol	Short GnRH-a protocol	hCG (10,000 U) when at least 1 of the follicles = 16-18 mm	Total r-FSH dose: GnRH-a < GnRH-ant, $p < 0.005$ No. of mature oocytes retrieved: GnRH-a > GnRH-ant, $p < 0.05$ No. of top-quality embryos transferred: GnRH-a > GnRH-ant, $p < 0.005$ Fertilization rate: GnRH-a > GnRH-ant, $p < 0.01$ Implantation and PR: NS
Manno M et al (2009)	23 (-ant)/ 52 (long)/ 68 (flare-up)	Multiple-dose fixed protocol	Long GnRH-a protocol	u-hCG (10,000 IU) or r-hCG (6500 IU) 36 hours before oocyte pick up	Total pregnancies/oocyte pick-up: Flare-up depot > antagonist, $p = 0.04$ Implantation rate: Flare-up depot > antagonist, $p = 0.029$ Fertilization failure: Flare-up depot < antagonist, $p = 0.04$ Clinical PR/oocyte pick-up and Clinical PR/Embryo Transfer: NS
			New flare-up depot GnRH-a protocol		NS differences between the GnRH-ant protocol and the e long protocol
Sbracia M et al (2009)	281/283	Multiple-dose flexible protocol	Long GnRH-a protocol	hCG (10,000 IU) when plasma E_2 > 800 and < 3500 pg/mL and at least 3 follicles > 16 mm	Cycles with transfer (%): GnRH-a > GnRH-ant, $p < 0.01$ E_2 levels (hCG day): GnRH-a > GnRH-ant, $p < 0.01$ Oocytes yielded: GnRH-a > GnRH-ant, $p < 0.01$ Implantation rate: GnRH-a > GnRH-ant, $p < 0.02$ PR/cycle: GnRH-a > GnRH-ant, $p < 0.01$ PR/transfer: GnRH-a > GnRH-ant, $p < 0.01$ Total r-FSH dose, Embryos transferred, abortion rate: NS

Table conotinued

Table II *(Continued)*. Italian studies on the use of GnRH antagonists in controlled ovarian hyperstimulation for *in vitro* fertilization in poor responders

Publication	Population (n/n)	GnRH-ant protocol	Other protocol	Trigger for ovulation	Main results
D'Amato G et al (2004)	85/60	r-FSH/CC/ GnRH-ant (Multiple-dose flexible protocol)	Long GnRH-a protocol	hCG when follicles ≥ 18 mm	Cancellation rates: GnRH-a > GnRH-ant, $p < 0.01$ Total r-FSH dose: GnRH-ant > GnRH-a, $p < 0.01$ E_2 levels (days 5 and 9): GnRH-an t > GnRH-a, $p < 0.01$ No. of oocytes retrieved: GnRH-ant > GnRH-a, $p < 0.01$ No of type 1 embryos: GnRH-ant > GnRH-a, $p < 0.01$ PR, implantation rate, NS
Alviggi C et al (2006)	67/66	Multiple-dose flexible protocol + r-LH	Short GnRH-a protocol	hCG (10,000 IU) when at least 1 follicle = 18-20 mm	E_2 concentration (day 5): GnRH-a > GnRH-ant, $p < 0.05$ No. of mature oocytes: GnRH-ant > GnRH-a, $p < 0.05$ No. of 2PN oocytes: GnRH-ant > GnRH-a, $p < 0.05$ PR, implantation rate, NS

hCG: human chorionic gonadotropin; CC: clomiphene citrate; E_2: estradiol; PR: pregnancy rate; NS: not significant; 2PN: two pronuclei.

the standard long protocol in poor responder patients undergoing IVF or ICSI[38]. In this trial the GnRH-ant protocol (375 IU of r-FSH administered from day 2 of the cycle plus 0.25 mg of Cetrorelix added when two follicles had reached 14 mm of diameter until the day of hCG administration) was compared to the standard long GnRH-a protocol (leuprorelin 3.75 mg from day 23 of the cycle and 375 IU of r-FSH daily from day 3 of the subsequent cycle). A statistically significant difference in terms of duration of ovarian stimulation (9.8 ± 0.8 vs. 14.6 ± 1.2), number of ampoules used (49.3 ± 4.3 vs. 72.6 ± 6.8), number of oocytes retrieved (5.6 ± 1.6 vs. 4.3 ± 2.2) and fertilized (3.8 ± 0.2 vs. 2.3 ± 0.23), number of follicles > 15 mm on the day of hCG administration favored the GnRH-ant group. In accordance to the above-mentioned meta-analysis[12], both the rate of cycle cancellation and the miscarriage rate did not differ significantly between the groups, but both parameters showed a trend towards better results in the GnRH-ant group. Despite no significant difference in either clinical pregnancy rate per woman (17% vs. 7% per embryo transfer) or in ongoing pregnancy rate could be observed, the latter was higher in the antagonist group. As to the works showing that GnRH-ant administration is of no benefit in poor responders, Malmusi et al randomized prospective trial[39] demonstrated an impairment in both the stimulation parameters and the clinical outcome in ICSI candidates treated with a GnRH-ant protocol compared to GnRH-a flare-up regimen. The flare-up protocol seemed to be more effective in terms of number of FSH ampoules administered, number of mature oocytes (3.2 ± 1.5 vs. 1.7 ± 1.2), number of oocytes retrieved (3.5 ± 1.4 vs. 2.5 ± 1.2), fertilization rate (84% vs. 63%) and top-quality embryos (1 ± 1 vs. 0.3 ± 0.6) transferred. The implantation and pregnancy rate were similar in the two groups (12.5 vs. 9% and 25% vs. 21.4%, respectively). More recently, also Manno et al[37], reached similar conclusions in a retrospective study, in which they compared the efficacy of different protocols in poor responders (GnRH antagonist and long depot protocols with flare-up depot protocol using GnRH agonist higher dose - triptorelin 3.75 mg 20 days before or just the day before the start of COH, respectively). The differences between the flare-up depot and the antagonist groups in total pregnancies for oocyte pick up (10/68, 14.7% vs. 0/18, 0%) and implantation (11/70, 15.7% vs. 0/20, 0%) rates, and fertilization failure (8/53, 15% vs. 6/18, 33%) were statistically significant. The clinical pregnancy rate per embryo transfer (8/45, 17.7% vs. 0/12, 0%) was higher with the flare-up protocol, though not statistically significant. These findings are in accordance with a meta-analysis by Franco et al[40] comparing GnRH-a (long and

flare-up protocols) and GnRH-ant in poor responders. When the GnRH-ant regimen was compared to the long and flare-up protocols together, no significant difference was reported in terms of cycle cancellation rate, number of mature oocytes, clinical pregnancy rate per cycle initiated/per oocyte retrieval/ per embryo transfer. However, the number of oocytes retrieved varied according to the protocols compared. When the GnRH-ant protocol was set against the GnRH-a long protocol, the former was related to a significant higher number of retrieved oocytes. On the contrary, the comparison between the GnRH-ant and flare-up protocols favored the latter in terms of number of retrieved oocytes. Finally, Sbracia et al study[26] was restricted to older patients[26], in which GnRH-ant administration was compared to GnRH-a long protocol. A significant impairment in the cycle outcome (lower pregnancy rate per cycle, lower pregnancy rate per transfer, lower implantation rate, decrease in oocyte yield) was recorded in the GnRH-ant group. A possible explanation for these negative results can be inferred from the process of follicular recruitment in older patients. In fact in such patients the cohort of growing follicles has already been recruited and selected, thus GnRH-ant administration in the mid-luteal phase has no effect on the number of developing follicles recruited. On the contrary, the long protocol may increase the size of the follicle cohort recruited per cycle thanks to the flare-up effect in the luteal phase and allow additional follicles to enter the cohort of stimulated follicles, thanks to the prolonged ovarian stimulation[26].

Both the studies carried out by Sbracia et al and Manno et al report that GnRH-ant administration is associated with a lower PR and with a reduction in the oocyte yield. The reduction in PR could be due to a negative effect on follicular growth and oocyte development, resulting in an impairment in the quality of the oocytes retrieved[37,39]. A possible solution is represented by a novel protocol combining high dose r-FSH, clomiphene citrate (CC) and GnRH-ant. In such regimen CC administration in the early follicular phase stimulates gonadotropin release by the pituitary gland and it promotes aromatase activity in the granulose cells, increasing E_2 levels. Furthermore, the GnRH-ant is started when follicular development is almost complete (diameter of the leading follicle = 16 mm) thus it should not produce detrimental effects on follicular development and its action should be restricted to the prevention of premature LH surge. Finally, higher doses of r-FSH counteract the effect of GnRH-ant/r-FSH/CC combined treatment on cancellation rate. According to D'Amato et al[41], this regimen increases oocyte retrieval and PR in poor responders and increases PR in older patients to values comparable to younger ones. Alternatively, a flexible protocol has been proposed by De Placido et al[36]. This regimen modulates the decline in endogenous gonadotropins thanks to the progressive increase in the GnRH-ant dose and to the addition of LH. This protocol was applied to patients at risk of poor ovarian responsiveness, in which a significant increase in the number of mature oocytes as well as in E_2 levels on the fifth day of stimulation was recorded. This improvement in oocyte quality was probably due to a more physiological LH environment during follicular maturation[36]. Acevedo et al[42] work confirmed the efficacy of a similar flexible protocol in increasing the number of mature oocytes. In this study a trend towards higher clinical pregnancy rates, and pregnancy/transfer was also recorded, though it did not reach statistical significance.

In conclusion, the proper stimulation protocol for poor-responder patients has not been identified yet. The use of GnRH-ant in this category of patients is still controversial, though novel protocols including these drugs seem promising. Further studies are needed to evaluate the effect of GnRH-ant in poor responders as well as to assess the efficacy of the alternative protocols proposed.

GnRH Antagonists in Patients at High Risk of OHSS (High Responders)

OHSS is the most fearful complication of COH during cycles of IVF or ICSI, due to its potential fatal outcome. It may occur after triggering ovulation by hCG and is worsened by the onset of pregnancy. Many factors favor the occurrence of OHSS including age, body mass index (BMI) and polycystic ovary syndrome (PCOS). What is more, even the stimulation parameters can influence the onset of OHSS. In fact the use of exogenous hCG either to induce ovulation or for the luteal phase supplementation, as well as the stimulation regimen chosen (gonadotropin administration is related to an higher risk than CC) and the amount of gonadotropin administered should be taken into account as potential risk factor for OHSS[43].

Several studies comparing the incidence of OHSS in GnRH-a and GnRH-ant protocols

showed a reduced incidence of OHSS in the GnRH-ant group, probably because of the shorter duration of stimulation and the lower amount of GT ampoules required[9]. The two main Italian studies dealing with GnRH-ant administration in high responders are presented in Table III. According to international literature, a prospective, multicentre, comparative study performed by Ragni et al[43] demonstrated the efficacy of GnRH-ant in preventing OHSS. The safety of a GnRH-ant protocol was tested in 87 selected patients who had already been administered a GnRH agonist mid-luteal long protocol in a previous IVF/ICSI attempt, in which they had experienced OHSS or had been at risk of OHSS. Both the rate of cancelled cycles due to OHSS risk (56.3% *vs* 32.2%) and the incidence of moderate or severe OHSS/initiated cycle (27.6% *vs* 11,5%) were significantly lower in the GnRH-ant group. This result could probably be attributed to the shorter duration of the stimulation protocol and to the lower amount of gonadotropin used in the antagonist cycles, resulting in a lower number of follicles and lower E_2 levels on the day of hCG administration. Moreover, GnRH-ant administration was linked to an increased percentage of oocytes retrievals (67.8% vs. 43.7%) and embryo transfers (96.6% vs. 76.3%) per initiated cycles.

In conclusion, the Authors reported that GnRH antagonists: i) reduce the incidence of OHSS and the number of assisted fertilization cycles cancelled because of the risk of OHSS in high responder patients; ii) increase the oocyte retrievals and embryo transfer rates in this group of patients. As to the safety, an additional advantage of the GnRH-ant protocol is the possibility to administer a GnRH-a instead of hCG for ovulation triggering. When this procedure is performed, an aggressive luteal support should be used to counteract the adverse effect of GnRH-a on the luteal phase. Besides the higher safety of the GnRH-ant protocol, its efficacy (i.e. the possibility to increase PR) in high responders should also be considered. In fact, the extremely high levels of E_2 observed in high responders impair both embryo quality and endometrial receptivity, and thus threat embryo implantation. Additionally, endometrial gene expression is altered during COH, compared to the natural cycle, resulting in a possible embryo-endometrial asynchrony. It has been suggested that an antagonist protocol may more closely resemble the natural cycle at endometrial level in terms of gene expression. Since GnRH-ant reduce also the risk of OHSS, such a protocol could be both safer and more effective in high responders. In a retrospective study performed by Manno et al[44] the cycles were divided on the

Table III. Italian studies on the use of GnRH antagonists in controlled ovarian hyperstimulation for *in vitro* fertilization in high responders.

Publication	Population (n/n)	GnRH-ant protocol	Other protocol	Trigger for ovulation	Main results
Ragni G et al (2005)	87/87	Multiple-dose flexible protocol	Long GnRH-a protocol	u-hCG when the leading follicle ≥ 18 mm	Cancellation rate: GnRH-a > GnRH-ant, $p < 0.001$ OHSS rate: GnRH-a > GnRH-ant, $p < 0.006$ No. of follicles > 10 mm: GnRH-a > GnRH-ant, $p < 0.001$ E_2 levels (hCG day): GnRH-a > GnRH-ant, $p < 0.001$
Manno M et al (2011)	200/200	Multiple-dose fixed protocol	Long GnRH-a protocol	hCG (6500 IU)	Cycles with peak E_2 > 3000 pg/ml: Total PR: GnRH-a > GnRH-ant, $p = 0.002$ Clinical PR: GnRH-a > GnRH-ant, $p = 0.0007$ Implantation rate: GnRH-a > GnRH-ant, $p = 0.0006$ Cycles with peak $E_2 \leq 3000$ pg/ml: Total PR: NS Clinical PR: NS Implantation rate: GnRH-ant > GnRH-a, $p = 0.009$

hCG: human chorionic gonadotropin; E_2: estradiol; PR: pregnancy rate; NS: not significant.

grounds of the E_2 levels recorded. Although in cycles with $E_2 \leq 3000$ pg/ml only a trend favoring the GnRH-a was reported, in cycles with $E_2 > 3000$ pg/ml the implantation rate was significantly higher in GnRH-ant group. This work suggests that an increase in pregnancy rate with increasing peak E_2 level can be observed with antagonist but not long protocol. Given the comparable quality of transferred embryos, these data could suggest a different effect of the two protocols on endometrial receptivity at least at extreme ovarian response. In synthesis, a GnRH-ant protocol using a GnRH-a for ovulation triggering and including an aggressive luteal support shows interesting PR and minimizes or avoids altogether the risk of both early and late OHSS.

GnRH Antagonists in PCOS Patients

The ovarian stimulation in PCOS patients represents a matter of concern. This gynecological disease, affecting about 6-15% reproductive age women, is characterized by chronic anovulation and hyperandrogenism. Furthermore, PCOS is a condition of relative insulin resistance, resulting in chronic hyperinsulinemia, abnormal ovarian androgen metabolism and altered gonadotropin responses[45]. Insulin sensitizer agents, such as metformin, and weight loss reduce the levels of LH and insulin and are sometimes sufficient to restore ovulation. Otherwise, ovarian stimulation should be performed by CC, as first line therapy[46]. If CC is unsuccessful, rFSH should be administered at low dose according to an individualized regimen, in order to avoid OHSS and multiple pregnancy (MP). In fact multifollicular development, and the subsequent increased risk of both OHSS and MP, represent the main difficulty of ovulation induction in these patients[47]. GnRH-a are not commonly used in standard protocols for ovulation induction in PCOS because of the higher amount of gonadotropin needed, the greater prevalence of multiple follicle development and the higher risk of both OHSS and MP. On the contrary, GnRH-ant represent a valid alternative to GnRH-a to prevent premature LH surge in PCOS patients. In fact these drugs reduce the duration of the stimulation as well as the risk of OHSS[48] and represent a safer way to induce final oocyte maturation. These findings result from a wide review[12] comparing GnRH-ant and GnRH-a both in patients considered overall and in PCOS-affected. GnRH-ant reduced the risk of OHSS in both categories and granted comparable ongoing pregnancy rates and clinical pregnancy rates in PCOS patients. Thus, the GnRH-ant regimen shows a similar efficacy and a greater safety than the traditional protocol in PCOS patients and hence it might be considered the protocol of choice in this category. The two major Italian studies that have addressed the topic are presented in Table IV.

A recent study was performed to evaluate the effect of GnRH-ant on gonadotropin and ovarian steroid secretion in the early follicular phase[47]. In this work, a group of women affected by PCOS was compared to a control group made up of normal ovulatory patients. Serum gonadotropin, E_2, testosterone (T), 17-Hydroxyprogesterone (17-OH-P), and androstenedione plasma levels were evaluated at baseline and 12 and 24 hours after each daily injection. These hormones were also assayed on days 10, 12, and 14 of the menstrual cycle. The administration of GnRH-ant resulted in a higher suppression of androgen and gonadotropin levels in PCOS patients, though it did not affect E_2 levels significantly. Thus the crucial role of E_2 on endometrium and on oocyte development is preserved. The higher pituitary responsiveness to GnRH-ant observed in PCOS patients should be ascribed to a higher sensitivity of their pituitary receptors. The excellent response to Cetrorelix documented in this study confirms the major role of GnRH-ant as first choice drug to control LH and androgen secretion in PCOS patients. The efficacy of a GnRH-ant based protocol is further improved by a pre-treatment with metformin, as demonstrated by Doldi et al[46]. In this study forty PCOS patients were divided into two groups. Women belonging to the study group were given metformin for 2 months and were then stimulated with rFSH. GnRH-ant administration was started when the leading follicle reached 14 mm diameter on ultrasound scan. Women belonging to the control group underwent the same stimulation protocol but they were not pretreated with metformin. The use of metformin led to an improvement in the outcome of ovarian stimulation in FIVET cycles in terms of reduction in the dose of gonadotropins required, serum E_2 levels on the day of hCG administration, incidence of OHSS and number of cancelled cycles. An increase in the mean number of mature oocytes was also recorded, when metformin was given. Even though these studies demonstrated the excellent response to GnRH-ant and the additional benefit of a pre-treatment with metformin in PCOS patients, more data are needed to confirm these findings.

Table IV. Italian studies on the use of GnRH antagonists in controlled ovarian hyperstimulation for *in vitro* fertilization in pcos patients.

	Materials and Methods			
Publication	**Study Group**	**Control Group**	**Treatment**	**Main results**
Sagnella F et al (2009)	PCOS affected (n=10)	Patients with normal ovulation (n=10)	Daily GnRH-ant administration administration at days 10, 12, and 14 of the menstrual cycle	Suppression of FSH and LH for the entire l ength of therapy: PCOS > controls, $p < 0.05$ LH recovery secretion: PCOS > controls, $p < 0.05$ Suppression of Androstenedione and 17-OH-Progesteron: NS
Doldi N et al (2006)	PCOS affected (n=20) Standard short GnRH-ant protocol for ovarian stimulation with 2 month-long metformin pretreatmentt	PCOS affected (n=20) Standard shor GnRH-ant protocol for ovarian stimulation		Total r-FSH dose: GnRH-ant+ M < GnRH-ant, $p < 0.05$ Serum E_2 (hCG day): GnRH-ant + M < GnRH-ant, $p < 0.05$ No. of mature oocytes: GnRH-ant+ M > GnRH-ant, $p < 0.05$ No. of cancelled cycles: GnRH-ant+ M < GnRH-ant, $p < 0.05$ OHSS rate: GnRH-ant+ M < GnRH-ant, $p < 0.05$ No. of follicles ≥ 14 mm, No. of oocytes retrieved, Duration of the stimulation: NS

FSH: follicle-stimulating hormone; LH: luteinizing hormone; E_2: estradiol; PR: pregnancy rate; NS: not significant.

GnRH Antagonists in Obese Patients

According to several studies, high BMI adversely affects ART outcome. In fact assisted reproduction cycles in high-BMI-patients require higher doses of gonadotropins and longer stimulation periods and are characterized by a higher incidence of follicular asynchrony and cancellation rate[49-51]. On the contrary, some Authors did not report any significant difference in ovarian response to stimulation in high-BMI-patients[49,52-53]. Literature shows conflicting results also regarding pregnancy and live-birth rates in obese women undergoing ART cycles. Some studies reported lower pregnancy and live-birth rates, lower implantation rates, increased obstetric complications and higher miscarriage rate. A meaningful study in this respect was conducted by Marci et al[49]. In this prospective study the influence of BMI on IVF/ICSI outcome was evaluated in 463 women. The patients were assigned either to a GnRH-a group or to a GnRH-ant group. Besides, the sample was further divided into two subgroups on the basis of their BMI (subgroup A, BMI < 25 kg/m^2; subgroup B, BMI ≥ 25 kg/m^2) in order to examine the effect of this parameter on the cycle outcome. The results of the study were analyzed both according to the BMI and to the stimulation regimen used. As far as BMI is concerned, the total amount of GT used proved to be higher and the stimulation longer in the subgroup B, irrespectively of the stimulation protocol used. However, subgroup A and B did not differ significantly either in the number of oocytes retrieved or in the number of embryos transferred or in the clinical pregnancy rate. Patients were further analyzed according to the stimulation protocol used. Women treated with GnRH-a required a significantly higher amount of GT and a longer stimulation period, irrespectively of their BMI. In subgroup A, the number of embryos obtained and transferred per starting patients resulted higher in patients administered GnRH-a. In subgroup B, the number of embryos transferred and the clinical pregnancy rate did not differ significantly on the grounds of the stimulation protocol used. The cancellation rate was higher in the agonist group compared to the antagonist group, and in obese patients compared with normal-weight patients. The clinical pregnancy rate was higher in patients with normal BMI. On the grounds of these results, ovarian stimulation with GnRH-ant can be considered an

efficient and acceptable treatment for both normal and high BMI patients. In fact, a GnRH-ant regimen is as effective as the traditional stimulation protocol and shows further advantages, such as a reduced amount of rFSH needed, a shorter duration of the stimulation period and a lower risk of OHSS. Even though these results sound convincing, the impact of GnRH-ant on obese women must be further investigated.

GnRH Antagonist and Follicular Environment

Ovarian physiology is a complex network of regulatory mechanisms involving steroid hormones, gonadotropins, growth factors and cytokines[54]. The molecules commonly associated with the inflammatory cascade, including prostaglandins, leukotrienes, bradykinin, histamine, platelet activating factors and various cytokines, were found in the ovary. In fact, cytokines are known to modulate ovarian function, gonadal steroid secretion, corpus luteum function, embryo development and implantation[55-56]. Therefore, it is important to understand how different ovarian stimulation protocols, using GnRH agonist or antagonist, affect the follicular environment and the delicate balance of the process that leads to pregnancy during IVF cycles. Table V summarizes the findings of the main Italian studies dealing with this topic.

During COH the multiple follicle recruitment is associated with an ovarian phlogistic process[57]

Table V. Italian studies on the effect of GnRH antagonists on follicular environment.

Publication	Population (n/n)	GnRH-ant protocol	Other protocol	Trigger for ovulation	Main results
Fornaro F et al (2007)	36/37	Multiple-dose flexible protocol	Long GnRH-a protocol	hCG (10,000 IU) when at least 3 follicles ≥ 18 mm	E2 (hCG day): GnRH-a > GnRH-ant, $p < 0.001$ No. of follicles ≥ 15 mm: GnRH-a > GnRH-ant, $p < 0.01$ No. of oocytes retrieved: GnRH-a > GnRH-ant, $p < 0.03$ Levels of sICAM-1 in small and large follicles: GnRH-a > GnRH-ant, $p < 0.05$ Fertilization rate, Implantation rate, PR/attempt, levels of sVCAM-1 in small and large follicles: NS
Ferrari B et al (2006)	30/30	Multiple-dose flexible protocol	Long GnRH-a protocol	hCG (10,000 IU) when the oocytes' maturation parameters were achieved	FF VEGF concentrations: GnRH-ant > GnRH-a, $p < 0.001$ LH levels: GnRH-a > GnRH-ant, $p < 0.001$ E_2 Serum and FF levels: GnRH-a > GnRH-ant, $p < 0.05$ FF androstenedione levels: GnRH-a > GnRH-ant, $p < 0.05$ PR, NS
Centurione L et al (2010)	11/10	Multiple-dose fixed protocol	Long GnRH-a protocol	hCG (10,000 IU) when at least 3 follicles ≥ 18 mm	Large/pale cells %: GnRH-a > GnRH-ant, $p < 0.05$ Small/dark cells %: GnRH-ant > GnRH-a, $p < 0.05$ Total r-FSH dose: GnRH-a > GnRH-ant, $p < 0.04$ Serum E2 (hCG day) GnRH-a > GnRH-ant, $p < 0.01$ Fertilization, Pregnancy, Implantation rate NS
Dell'Aquila ME et al (2009)	13/31	–	–	hCG in; GnRH-a group hCG/GnRH-a in GnRH-ant group	Rate of polarized mythocondria distribution: GnRH-a > GnRH-ant, $p < 0.05$ Mature oocytes (%): NS

hCG: human chorionic gonadotropin; E_2: estradiol; PR: pregnancy rate; FF: follicular fluid; NS: not significant.

and with a change in the expression of adhesion molecules, such as the intercellular adhesion molecule-1 (ICAM-1) and the vascular cell adhesion molecule-1 (VCAM-1). The levels of these factors, in their soluble form (sICAM-1 and sVCAM-1), might mirror different aspects of the follicular development process. Intrafollicular sICAM-1 content may predict ovarian response, since its secretion is related to the granulosa-lutein cells activity[58]. Intrafollicular sVCAM-1 indicates the degree of follicular luteinisation, and acts as a proangiogenic factor in the late phases of follicle development. On the grounds of these observations, a randomized prospective study was performed to compare the effects of either a GnRH-a flare-up protocol or GnRH-ant-based stimulation regimen on follicular fluid (FF) levels of sICAM-1 and sVCAM-1[59]. The content of sICAM1, sVCAM1, E_2, and progesterone (P) were measured according to the stage of follicular development in FF aspirated from small and large follicles. As to the cycle outcome parameters, the number of follicles with a diameter ≥15 mm resulted significantly higher in GnRH agonist protocol. No significant differences were reported in the number of mature oocytes and the number of top-quality embryos, as well as in the fertilization, implantation and pregnancy rates. Focusing on the levels of adhesion molecules in the FF, the follicular content of sICAM-1 was positively related to the number of follicles of ≥15 mm and to the number of oocytes retrieved in both study groups. Thus ICAM-1 can be considered a biochemical marker of ovarian response to COH. Since FF concentrations of sICAM-1 were higher in GnRH-a group irrespectively of the follicle size, this parameter could mirror the better cycle outcome observed in this group. FF levels of sVCAM-1 did not differ significantly on the grounds of the stimulation protocol used, though they varied according to the size of the follicles. Since higher levels of VCAM-1 were detected in larger follicles, the expression of this glycoprotein probably increases with follicular growth. Moreover, given the positive correlation between VCAM-1 and P values in both follicular classes, VCAM-1 probably mirrors the degree of follicular maturation and luteinisation. Given the positive correlation between FF levels of VCAM-1 and VEGF, a cooperation between these two factors in regulating the human luteinisation process can be supposed. VEGF is a potent growth factor implicated in neoangiogenesis from pre-existing microvessels[60]. Hypoxia and hypoglycaemia stimulate the expression of VEGF, providing blood supply to tissues that were previously deprived of blood circulation or were hypoxic. VEGF is produced by granulose and theca cells and its expression in the pre-ovulatory follicle and in corpus luteum cells is controlled by gonadotropins (FSH, LH, and hCG). This factor plays a major role in angiogenesis at ovarian level. In fact it grants peripheral blood supply to pre-antral follicles and it helps create and maintain the vascularisation of the corpus luteum. High FF-VEGF levels are reported in older patients[61], they are predictive of poor ovarian response and they are related to suboptimal embryo development and to poor conception rates[62-63]. Thus, FF-VEGF is an excellent marker of cell suffering because the cells presumably impaired by locally reduced oxygen level produce and paracrinally release this growth factor in the interstitial fluid. On the grounds of these observations Ferrari et al[64] examined the concentration of VEGF in the follicular fluid (FF-VEGF) of women undergoing IVF, who were administered either a GnRH-ant or a GnRH-a. Furthermore, hormone concentrations were evaluated both in FF (E_2, androstenedione) and in serum (E_2, LH). In the GnRH-ant group, the recorded levels of FF-VEGF were significantly higher (2906 ± 1558.5 vs. 1598.5 ± 612.16 pg/ml), whereas follicular fluid estradiol and androstenedione levels were significantly reduced (621 ± 435 vs. 1146 ± 593; 78 ± 31 vs. 136 ± 55). The pregnancy rate was higher, though not statistically significant, in GnRH-a group (23.3% vs. 16.6%).

In conclusion, the increased VEGF secretion reflects a ''hostile'' follicular environment, in which the deep suppression of LH does not allow adequate steroidogenesis. The resulting hypoestrogenic environment may compromise cell function -since E_2 plays an important role in oocyte maturation- and may justify the lower PR observed in GnRH-ant group.

Furthermore, mounting evidence suggests that the analogues of GnRH may display a direct effect on human ovaries, besides their pituitary action[65-66]. In fact GnRH receptors have been found in granulose cells (GCs), prompting researches on the effect of GnRH-a and GnRH-ant on follicular development and steroidogenesis. Centurione et al examined the morphological characteristics of granulose cells obtained from women undergoing either a GnRH-a or a GnRH-ant stimulation protocol. On the grounds of the morphological features observed at microscope, two cell populations were detected: pale cells (late stages of fol-

licular luteinisation) and dark cells (early stages of follicular luteinisation). In the GnRH-a group a significantly higher number of pale cells was reported, while in the GnRH-ant group dark cells were prevalent. Dark cells characteristics were suggestive of a lower steroidogenic activity; additionally, the higher number of lipid droplets reported in these cells could reflect a non-functional status, in which unreleased steroids are stored at intracellular level. Thus, dark cells could stand for metabolically blocked cells, possibly destined to apoptosis[67]. Since the incidence of granulose cells' apoptosis has been related to ART outcome, Giampietro et al[68] compared the levels of apoptosis in GCs from patients undergoing ART, whether treated with Triptorelin or Cetrorelix and evaluated a possible hormonal mediated influence on apoptosis. Even though the concentration of androgen, E_2 and P in the follicular fluid was lower in GnRH-ant treated patients, the levels of apoptosis observed in the two groups were comparable. Thus the severe impact of GnRH-ant on ovarian steroidogenesis was confirmed, but no correlation was detected between steroid levels in FF and GC apoptosis.

Finally, a randomized study conducted by Dell'Aquila et al[3] was the first to analyze the effects of different stimulation regimens on human oocyte energy status in terms of mitochondrial distribution patterns. The distribution of active mitochondria within the oocyte reflects the energy and ion requirement necessary for key cellular cycle events, like oocyte maturation, fertilization, and embryo development. Fluorescent staining and confocal laser scanning microscopy were performed on 225 supernumerary mature oocytes after the use of either GnRH agonist or GnRH antagonist. Although in both groups fluorescence intensity did not vary according to the mitochondrial distribution pattern, a higher fluorescence intensity was reported in oocytes with polarized and large granules configurations in GnRH-ant treated patients. This phenomenon may be detrimental as hypothesized beforehand. Thus the Authors concluded that GnRH antagonists could induce mitochondrial hyperactivity, which is held responsible for an impairment in the oocytes' quality.

In synthesis many studies dealt with the effect of GnRH-ant on follicular environment: most of them confirmed the severe impact of these drugs on ovarian steroidogenesis[64,67-68]. Even though the reduction in ovarian steroidogenesis does not impair ART outcome by influencing the process of GCs apoptosis[68], the resulting hypoestrogenic environment can adversely affect follicular development[64]. The levels of adhesion molecules such as ICAM-1 allow to predict ovarian response to stimulation, whereas VCAM-1 only reflects the degree of follicular luteinisation. Finally, even oocyte quality can be compromised in GnRH-ant protocols because of mitochondrial hyperactivity[3].

GnRH-Antagonists in Intrauterine Insemination (IUI)

Nowadays, IUI is one of the most widely used fertility treatment in clinical conditions such as idiopathic infertility, mild endometriosis, or mild to moderate male factor infertility. The association with COH, in particular with gonadotropins, increases pregnancy rate when compared to unstimulated cycles[13]. Yet stimulated cycles are characterized by a sudden rise in E_2 level, favouring the untimely release of LH. This hormone induces a change in the steroidogenic activity of granulose cells, which is reflected in P production. This premature luteinisation (PL), due to endogenous LH surge, is associated with a less favorable outcome because of poor oocyte quality and decreased fertilization and implantation rate. Thus it is a common cause of cycle cancellation and ensuing patient distress. Even though the efficacy of GnRH-ant in the prevention of premature luteinisation has been widely confirmed[5-6], the impact of these drugs on PR is still controversial (Table VI).

In fact, some works report that GnRH-ant addition does not increase PR in COH/IUI cycles. Crosignani's[16] study is very meaningful in this respect, because it is a randomized controlled trial involving a large sample and pooling data from different countries. Two-hundred and ninety-nine women were allocated either in the GnRH-ant group or in the control group. Controls were treated only with 50 IU recombinant follicle stimulating hormone (r-FSH) starting on day 3 of the menstrual cycle, while in the GnRH-ant group 0.25 mg daily dose of Ganirelix was added when a follicle with a mean diameter of 13-14 mm was visualized at ultrasound. The clinical pregnancy rates per initiated cycle (12.6% vs. 12.2%) and per completed cycle (14.5% vs. 13.8%) were similar in the two groups. On the grounds of these results the Authors stated that the additional administration of a GnRH-ant in COH-IUI does not improve pregnancy rate in COH-IUI. Since the effectiveness of these drugs in preventing premature LH surge has already been demonstrated, the lack of any improvement

Table VI. Italian studies on the use of GnRH antagonists in controlled ovarian stimulation for intrauterine insemination.

Publication	Population (n/n)	GnRH-ant protocol	Other protocol	Trigger for ovulation	Main results
Crosignani PG et al (2007)	148/151	Standard gonadotropin superovulated protocol (r-FSH) + GnRH-ant (Multiple-dose flexible protocol)	Standard gonadotropin superovulated protocol (r-FSH only)	hCG (5000 IU) when the leading follicle > 18 mm	Duration of stimulation: GnRH-ant > GnRH-a, $p = 0.02$ No. of follicles 16 mm, No. of follicles 11-15 mm, Clinical PR/started cycle, Clinical PR/completed cycle: NS
Allegra A et al (2007)	52/52	Gonadotropin superovulated protocol (r-FSH) + GnRH-ant (Multiple-dose flexible protocol)	Standard gonadotropin superovulated protocol (r-FSH only)	hCG (10,000 IU) when the leading follicle ≥ 18 mm	Total r-FSH dose: GnRH-a > GnRH-ant, $p = 0.009$ Rate of Premature LH surge: GnRH-a > GnRH-ant, $p < 0.0001$ Rate of PL: GnRH-a > GnRH-ant, $p = 0.001$ Mean values of LH and P: GnRH-a > GnRH-ant, $p < 0.0001$ Clinical PR: GnRH-ant > GnRH-a, $p = 0.017$ Duration of the stimulation, No. of follicles ≥ 16 mm: NS
Ragni G et al (2004)	32/34	Gonadotropin superovulated protocol (r-FSH daily) + GnRH-ant (Multiple-dose flexible protocol)	Gonadotropin superovulated protocol (r-FSH on alternate days) + GnRH-ant (Multiple-dose flexible protocol)	hCG (5000 IU) when the leading follicle > 18 mm	No. Follicles ≥ 16 mm and ≥ 11 mm: GnRH-ant+r-FSH (daily) > GnRH-ant + r-FSH (on alternate days), $p = 0.02$ Rate of mono-ovulation: GnRH-ant + r-FSH (on alternate days) > GnRH-ant + r-FSH (daily), $p = 0.06$ Clinical PR/initiated cycle: GnRH-ant + r-FSH (daily) > GnRH-ant + r-FSH (on alternate days), $p = 0.05$
Ragni G et al (2006)	621 (1259 cycles)	Gonadotropin superovulated protocol (r-FSH) + GnRH-ant (Multiple-dose flexible protocol)	Gonadotropin superovulated protocol (r-FSH) + GnRH-ant (Multiple dose flexible protocol)	hCG when the leading follicle ≥ 18 mm	Cancellation rate = 10.5% Cumulative PR/couple = 18.7% Incidence of twin pregnancies = 9.5% Incidence of high-order MP = 0 Live birth rate/initiated cycle = 7.0% Live birth rate/completed cycle = 7.8%
Ragni G et al (2001)	19/22	Gonadotropin superovulated protocol (r-FSH) + GnRH-ant (Multiple-dose flexible protocol)	Standard gonadotropin superovulated protocol (r-FSH only)	hCG when the leading follicle ≥ 18 mm	Mean duration of the luteal phase: GnRH-ant > GnRH-a ($p < 0.05$) E_2 concentration (day 6 after hCG): GnRH-a > GnRH-ant ($p < 0.05$) E_2/P ratio, NS

hCG: human chorionic gonadotropin; PR: pregnancy rate; NS: not significant; LH: luteinizing hormone; E_2: estradiol; P: progesteron; PL: premature luteinisation; MP: multiple pregnancy.

in PR remains unexplained. Probably, the benefits related to the prevention of the LH surge are balanced by the not well-understood detrimental effects of GnRH agonists or antagonists. In this regard, it is noteworthy that a modification of serum hormonal levels after initiation of GnRH antagonist has been reported[6]. Since the administration of a GnRH-ant is immediately followed by a deep suppression of LH release, it should be delayed so as to prolong the effect of endogenous LH on follicular growth. In Allegra et al work, the effect of endogenous LH on the growing follicles was maintained until precise criteria for Cetrorelix administration were met (E_2 levels

higher than 200 pg/ml, LH < 10 mUI/ml, P < 2 ng/ml and diameter of the leading follicle higher than 16 mm). This clinical decision has led to a significant increase in PR and to a parallel reduction in the incidence of premature luteinisation[7]. Besides Allegra's, many works reported a significantly higher PR when a GnRH-ant was added to a gonadotropin superovulated protocol[13,69-70].

Two hypotheses have been advanced to explain the higher PR related to GnRH-ant administration. First, the prevention of premature LH surge and subsequent premature luteinisation reduces cycle cancellation. Second, since the occurrence of untimely LH release is rare when a GnRH-ant is administered, exogenous gonadotropins administration can be continued until more ovulatory follicles are recruited. In accordance with the latter hypothesis, the higher PR achieved depends on a larger pool of mature follicles and does not represent a direct effect of the GnRH-ant[70]. However, in Allegra's study the number of mature follicles was similar in the groups and hence the higher PR recorded in the GnRH-ant group may be explained by the prevention of premature LH surge and premature luteinisation. Nevertheless, according to more recent studies[13], GnRH-ant act synergistically in preventing premature luteinisation and in increasing the pool of mature follicles on the day of hCG administration. Thus it can be speculated that such a protocol determines an increase in the risk of MP by increasing the number of mature follicles on the day of hCG administration. The safety of a GnRH-ant based protocol has been evaluated in literature. In a randomized prospective study[5], Ragni et al tried to detect the lowest effective dose of GT that may ensure both a decrease in multiple-birth risk and an acceptable pregnancy rate. For this purpose, the Authors compared two different protocols employing GnRH-ant combined either with a daily dose of r-FSH or with r-FSH administration on alternate days. No multiple pregnancies were detected in either group. The protocol based on daily r-FSH was associated with a lower rate of mono-ovulation (53.3% versus 78.8%), but also with a higher clinical pregnancy rate per initiated cycle (34.4% versus 5.9%). Thus, a stimulation protocol combining a daily dose of 50 IU of recombinant FSH with a GnRH antagonist may provide a good pregnancy rate without exposing women to a high risk of multiple pregnancies. The safety of such protocol was further clarified by a subsequent retrospective study carried out by the same Author. 1259 cycles were considered, in which strict cancellation criteria were adopted to further prevent multiple gestations. The clinical pregnancy rate per initiated cycle was 9,2% and the clinical pregnancy per completed cycle was 10.3%. The live-birth rates were 7.0% and 7.8%, respectively. Moreover, the incidence of twins was 9,5% and no high-order multiple pregnancy was observed. Thus, a protocol of 50 IU of recombinant FSH per day combined with the use of a GnRH antagonist and a policy of strict cancellation based on ultrasound criteria can be proposed to achieve a satisfactory pregnancy rate without a significant increase in the risk of high-order multiple pregnancies[15].

One more controversial topic is the necessity of luteal phase support in GnRH-ant based protocols. In a randomized study, Ragni et al[71] compared the luteal phase profile in GT-stimulated cycles with or without GnRH-ant. Serum LH was completely suppressed during the follicular phase only in the GnRH antagonist group, while the increase in P concentration during luteal phase was similar in the two protocols. Moreover PR were comparable in the two groups. Indeed, this study demonstrated that GnRH antagonists grant an effective suppression of LH peak without deleterious effects either on the luteal P concentration or on the duration of the luteal phase. Since a normal hormonal luteal profile in patients who are treated with recombinant FSH and GnRH antagonists has been documented, no luteal phase supplementation is needed.

In conclusion, despite conflicting results on PR, the effectiveness of GnRH-ant in preventing premature luteinisation has been widely demonstrated. In addition to that, further clinical advantages result from GnRH-ant administration. First, the risk of premature luteinisation is low when a GnRH-ant protocol is administered, thus no strict monitoring is needed[16]. Besides, this protocol is more flexible and can be adapted depending on hormonal and ultrasound findings[6]. Further studies are needed to clarify the mechanism of action of GnRH-ant as well as their effect on the prevention of premature LH surge and follicular development, so as to set the appropriate indications for a combined r-FSH/GnRH-ant stimulation protocol.

Conclusions

The classical GnRH-a long protocol is the most widely used in COH for ART. However, an

alternative regimen based on GnRH-ant has been recently introduced in clinical practice[12]. As competitive antagonists, these drugs display an immediate and quickly reversible effect and they avoid hormonal withdrawal side effects. Moreover this protocol shows undeniable advantages, including the shorter duration of the treatment, the lower amount of gonadotropin required, the shorter hormonal and ultrasound monitoring of patients, milder physical and emotional stress, and a lower risk of OHSS[11-12,72].

This review provides a comprehensive overview of the use of GnRH antagonist protocols applied both to IVF techniques and to IUI procedures in the Italian experience.

As to IVF, the heterogeneity of the studies presented mirrors the general disagreement in literature. This may be due to many factors: the different criteria of patient selection, the lack of uniformity in the definition of "normal-", "poor-", and "high-responders", the different protocols used in the ovarian stimulation, and the small number of women included in each study. Additionally, the experience in the use of GnRH antagonists may have influenced the results of the studies. Despite the greater safety of GnRH-ant, the reduced pregnancy rates reported in previous literature have hampered their introduction in clinical practice for many years. The use of GnRH-ant was traditionally restricted to selected patients, as "poor responders" and women at high-risk of developing OHSS such as PCOS affected and patients who had previously experienced OHSS. Nevertheless, the last Cochrane Systematic Review[11-12] has confirmed a greater safety of GnRH-ant and it has demonstrated an efficacy overall comparable to GnRH-a. These findings could prompt a trend to change from the standard agonist protocol to the antagonist protocol in all categories of patients. According to recent literature the therapeutic options should be expanded so as to customize the treatment on the grounds of the clinical characteristics and of the history of patients. Furthermore, the introduction of GnRH-ant in ART responds to the increasing need of an effective and safer management[73].

As to IUI, the capability of GnRH-ant to prevent premature LH surge and subsequent luteinisation has been widely demonstrated. Italian studies have shown the importance of a delicate hormonal milieu in which premature LH peak is deleterious but the deep suppression of such hormone during the early follicular phase impairs follicular development. In fact several studies demonstrated the negative effect of GnRH-ant on ovarian steroidogenesis. The resulting hypoestrogenic environment may compromise follicular growth and interfere with oocyte maturation, as demonstrated by the higher levels of VEGF. Thus, precise criteria for GnRH-ant administration, together with a strict hormonal and ultrasound monitoring should be adopted. The increase in PR reported by some studies depends on both a larger pool of mature follicles and on the prevention of the deleterious effect of LH on the immature follicles. Since the effectiveness and safety of this approach has been convincingly demonstrated, contradictory results could be attributed to the learning curve needed to optimize protocols with GnRH-ant. However, the impact of these drugs on follicular environment, ovarian steroidogenesis and endometrial receptivity should be carefully evaluated to lead to the proper management of GnRH-ant.

References

1) Lenton EA, Woodward B. Natural-cycle versus stimulated-cycle IVF: is there a role for IVF in the natural cycle? J Assist Reprod Gen 1993; 10: 406-408.
2) Fahy UM, Cahill DJ, Wardle PG, Hull MG. *In vitro* fertilization in completely natural cycles. Hum Reprod 1995; 10: 572-575.
3) Dell'Aquila ME, Ambruosi B, De Santis T, Cho YS. Mitochondrial distribution and activity in human mature oocytes: gonadotropin-releasing hormone agonist versus antagonist for pituitary down-regulation. Fertil Steril 2009; 91: 249-255.
4) Coccia ME, Comparetto C, Bracco GL, Scarselli G. GnRH antagonists. Eur J Obstet Gynecol Reprod Biol 2004; 115: S44-S56.
5) Ragni G, Alagna F, Brigante C, Riccaboni A, Colombo M, Somigliana E, Crosignani PG. GnRH antagonists and mild ovarian stimulation for intrauterine insemination: a randomized study comparing different gonadotrophin dosages. Hum Reprod 2004; 19: 54-58.
6) Lambalk CB, Leader A, Olivennes F, Fluker MR, Andersen AN, Ingerslev J, Khalaf Y, Avril C, Belaisch-Allart J, Roulier R, Mannaerts B. Treatment with the GnRH antagonist ganirelix prevents premature LH rises and luteinization in stimulated intrauterine insemination: results of a doubleblind, placebo-controlled, multicentre trial. Human Reproduction 2006; 21: 632-639.
7) Allegra A, Marino A, Coffaro F, Scaglione P, Sammartano F, Rizza G, Volpes A. GnRH antagonist-induced inhibition of the premature LH surge increases pregnancy rates in IUI-stimulated cycles. A prospective randomized trial. Hum Reprod 2007; 22: 101-108.

8) The Ganirelix dose-finding study group. A double-blind, randomized, dose-finding study to assess the efficacy of the gonadotrophin-releasing hormone antagonist ganirelix (Org 37462) to prevent premature luteinizing hormone surges in women undergoing ovarian stimulation with recombinant follicle stimulating hormone (Puregon). Hum Reprod 1998; 13: 3023-3031.

9) Albano C, Felberbaum RE, Smitz J, Riethmüller-Winzen H, Engel J, Diedrich K, Devroey P. Ovarian stimulation with HMG: results of a prospective randomized phase III European study comparing the luteinizing hormone-releasing hormone (LHRH)-antagonist cetrorelix and the LHRH-agonist buserelin. European Cetrorelix Study Group. Hum Reprod 2000; 15: 526-531.

10) European and Middle East Orgalutran Study Group. Comparable clinical outcome using the GnRH antagonist ganirelix or a long protocol of the GnRH agonist triptorelin for the prevention of premature LH surges in women undergoing ovarian stimulation. Hum Reprod 2001; 16: 644-651.

11) Al-Inany H, Aboulghar M. GnRH antagonist in assisted reproduction: a Cochrane review. Hum Reprod 2002; 17: 874-885.

12) Al-Inany HG, Youssef MA, Aboulghar M, Broekmans F, Sterrenburg M, Smit J, Abou-Setta AM. Gonadotrophin-releasing hormone antagonists for assisted reproductive technology. Cochrane Database Syst Rev 2011; 11: CD001750.

13) Bakas P, Konidaris S, Liapis A, Gregoriou O, Tzanakaki D, Creatsas G. Role of gonadotropin-releasing hormone antagonist in the management of subfertile couples with intrauterine insemination and controlled ovarian stimulation. Fertil Steril 2011; 95: 2024-2028.

14) Kosmas IP, Tatsioni A, Kolibianakis EM, Verpoest W, Tournaye H, Van der Elst J, Devroey P. Effects and clinical significance of GnRH antagonist administration for IUI timing in FSH superovulated cycles: a meta-analysis. Fertil Steril 2008; 90: 367-372.

15) Ragni G, Caliari I, Nicolosi AE, Arnoldi M, Somigliana E, Crosignani PG. Preventing high-order multiple pregnancies during controlled ovarian hyperstimulation and intrauterine insemination: 3 years' experience using low-dose recombinant follicle-stimulating hormone and gonadotropin-releasing hormone antagonists. Fertil Steril 2006; 85: 619-624.

16) Crosignani PG, Somigliana E. Intrauterine Insemination Study Group. Effect of GnRH antagonists in FSH mildly stimulated intrauterine insemination cycles: a multicentre randomized trial. Hum Reprod 2007; 22: 500-505.

17) Huirne JAF, Lambalk CB. Gonadotrophin-releasing hormone receptor antagonists. Lancet 2001; 358: 1793-1803.

18) Dal Prato L, Borini A. Use of antagonists in ovarian stimulation protocols. Reprod Biomed Online 2005; 10: 330-338.

19) Ludwig M, Felberbaum RE, Devroey P, Albano C, Riethmüller-Winzen H, Schüler A, Engel W, Diedrich K. Significant reduction of the incidence of ovarian hyperstimulation syndrome (OHSS) by using the LHRH antagonist Cetrorelix (Cetrotide) in controlled ovarian stimulation for assisted reproduction. Arch Gynecol Obstet 2000; 264: 29-32.

20) Roulier R, Chabert-Orsini V, Sitri MC, Barry B, Terriou P. Depot GnRH agonist versus the single dose GnRH antagonist regimen (cetrorelix, 3 mg) in patients undergoing assisted reproduction treatment. Reprod Biomed Online 2003; 7: 185-189.

21) Griesinger G, Felberbaum R, Diedrich K. GnRH antagonist in ovarian stimulation: a treatment regimen of clinicians' second choice? Data from the Germany national IVF registry. Hum Reprod 2005; 20: 2373-2375.

22) Kolibianakis EM, Collins J, Tarlatzis BC, Devroey P, Diedrich K, Griesinger G. Among patients treated for IVF with gonadotrophins and GnRH analogues, is the probability of live birth dependent on the type of analogue used? A systematic review and meta-analysis. Hum Reprod Update 2006; 12: 651-671.

23) Engel JB, Griesinger G, Schultze-Mosgau A, Felberbaum R, Diedrich K. GnRH agonist and antagonist in assisted reproduction: pregnancy rate. Reprod BioMed Online 2006, 13: 84-87.

24) Greco E, Litwicka K, Ferrero S, Baroni E, Sapienza F, Rienzi L, Romano S, Minasi MG, Tesarik J. GnRH antagonists in ovarian stimulation for ICSI with oocyte restriction: a matched, controlled study. Reprod BioMed Online 2007; 14: 572-578.

25) Tiboni GM, Palumbo P, Leonzio E, Gabriele E, Verna I, Giampietro F. Effectiveness of a low gonadotrophin-releasing hormone antagonist dose in preventing premature luteinizing hormone rise during controlled ovarian stimulation. Gynecol Endocrinol 2011; 27: 885-889.

26) Sbracia M, Colabianchi J, Giallonardo A, Giannini P, Piscitelli C, Morgia F, Montigiani M, Schimberni M. Cetrorelix protocol versus gonadotropin-releasing hormone analog suppression long protocol for superovulation in intracytoplasmic sperm injection patients older than 40. Fertil Steril 2009; 91: 1842-1847.

27) Depalo R, Lorusso F, Palmisano M, Bassi E, Totaro I, Vacca M, Trerotoli P, Masciandaro P, Selvaggi L. Follicular growth and oocyte maturation in GnRH agonist and antagonist protocols for *in vitro* fertilisation and embryo transfer. Gynecol Endocrinol 2009; 25: 328-334.

28) Kolibianakis EM, Zikopoulos K, Smitz J, Camus M, Tournaye H, Van Steirteghem AC, Devroey P. Elevated progesterone at initiation of stimulations associated with lower ongoing pregnancy rate after IVF using GnRH antagonists. Hum Reprod 2004; 19: 1525-1529.

29) Kolibianakis EM, Bourgain C, Papanikolaou EG, Camus M, Tournaye H, Van Steirteghem AC, Devroey P. Prolongation of follicular phase by delaying HCG administration results in a higher incidence

of endometrial advancement on the day of oocyte retrieval in GnRH antagonist cycles. Hum Reprod 2005; 20: 2453-2456.

30) Marci R, Caserta D, Lisi F, Graziano A, Soave I, Lo Monte G, Patella A, Moscarini M. *In vitro* fertilization stimulation protocol for normal responder patients. Gynecol Endocrinol 2013; 29: 109-112.

31) Casano S, Guidetti D, Patriarca A, Pittatore G, Gennarelli G, Revelli A. MILD ovarian stimulation with GnRH-antagonist vs. long protocol with low dose FSH for non-PCO high responders undergoing IVF: a prospective, randomized study including thawing cycles. J Assist Reprod Genet 2012; 29: 1343-1351.

32) Levi-Setti PE, Cavagna M, Bulletti C. Recombinant gonadotrophins associated with GnRH antagonist (cetrorelix) in ovarian stimulation for ICSI: comparison of r-FSH alone and in combination with r-LH. Eur J Obstet Gynecol Reprod Biol 2006; 126: 212-216.

33) Pandian Z, McTavish AR, Aucott L, Hamilton MP, Bhattacharya S. Interventions for 'poor responders' to controlled ovarian hyper stimulation (COH) in in-vitro fertilisation (IVF). Cochrane Database Syst Rev 2010; 20: CD004379.

34) Ubaldi FM, Rienzi L, Ferrero S, Baroni E, Sapienza F, Cobellis L, Greco E. Management of poor responders in IVF. Reprod Biomed Online 2005; 10: 235-246.

35) Ferraretti AP, La Marca A, Fauser BC, Tarlatzis B, Nargund G, Gianaroli L. ESHRE working group on Poor Ovarian Response Definition. ESHRE consensus on the definition of 'poor response' to ovarian stimulation for in vitro fertilization: the Bologna criteria. Hum Reprod 2011; 26: 1616-1624.

36) De Placido G, Mollo A, Clarizia R, Strina I, Conforti S, Alviggi C. Gonadotropin-releasing hormone (GnRH) antagonist plus recombinant luteinizing hormone vs. a standard GnRH agonist short protocol in patients at risk for poor ovarian response. Fertil Steril 2006; 85: 247-250

37) Manno M, Tomei F, Cervi M, Favretti C, Adamo V. Comparison of protocols efficacy in poor responders: differences in oocytes/embryos competence with different protocols, a retrospective study. Fertil Steril 2009; 91: 1431-1433.

38) Marci R, Caserta D, Dolo V, Tatone C, Pavan A, Moscarini M. GnRH antagonist in IVF poor-responder patients: results of a randomized trial. Reprod Biomed Online 2005; 11: 189-193.

39) Malmusi S, La Marca A, Giulini S, Xella S, Tagliasacchi D, Marsella T, Volpe A. Comparison of a gonadotropin-releasing hormone (GnRH) antagonist and GnRH agonist flare-up regimen in poor responders undergoing ovarian stimulation. Fertil Steril 2005; 84: 402-406.

40) Franco JG Jr, Baruffi RL, Mauri AL, Petersen CG, Felipe V, Cornicelli J, Cavagna M, Oliveira JB. GnRH agonist versus GnRH antagonist in poor ovarian responders: a meta-analysis. Reprod Biomed Online 2006; 13: 618-627.

41) D'Amato G, Caroppo E, Pasquadibisceglie A, Carone D, Vitti A, Vizziello GM. A novel protocol of ovulation induction with delayed gonadotropin-releasing hormone antagonist administration combined with high-dose recombinant follicle-stimulating hormone and clomiphene citrate for poor responders and women over 35 years. Fertil Steril 2004; 81: 1572-1577.

42) Acevedo B, Sanchez M, Gomez JL, Cuadros J, Ricciarelli E, Hernandez ER. Luteinizing hormone supplementation increases pregnancy rates in gonadotropin-releasing hormone antagonist donor cycles. Fertil Steril 2004; 82: 343-347.

43) Ragni G, Vegetti W, Riccaboni A, Engl B, Brigante C, Crosignani PG. Comparison of GnRH agonists and antagonists in assisted reproduction cycles of patients at high risk of ovarian hyperstimulation syndrome. Hum Reprod 2005; 20: 2421-2425.

44) Manno M, Cervi M, Zadro D, Fuggetta G, Adamo V, Tomei F. Different ART outcomes at increasing peak estradiol levels with long and antagonist protocols: retrospective insights from ten years experience. J Assist Reprod Genet 2011; 28: 693-698.

45) De Leo V, la Marca A, Petraglia F. Insulin-lowering agents in the management of polycystic ovary syndrome. Endocr Rev 2003; 24: 633-667.

46) Doldi N, Persico P, Di Sebastiano F, Marsiglio E, Ferrari A. Gonadotropin-releasing hormone antagonist and metformin for treatment of polycystic ovary syndrome patients undergoing in vitro fertilization–embryo transfer. Gynecol Endocrinol 2006; 22: 235-238.

47) Sagnella F, Apa R, Guido M, Villa P, Spadoni, V, Miceli F, Lanzone A. Suppression and recovery of gonadotropin and steroid secretion by a gonadotropin-releasing hormone receptor antagonist in healthy women with normal ovulation versus women with polycystic ovary syndrome in the early follicular phase. Fertil Steril 2009; 91: 1857-1863.

48) Griesinger G, Diedrich K, Tarlatzis BC, Kolibianakis EM. GnRH antagonists in ovarian stimulation for IVF in patients with poor response to gonadotrophins, polycystic ovary syndrome, and risk of ovarian hyperstimulation: a meta-analysis. Reprod Biomed Online 2006; 13: 628-638.

49) Marci R, Lisi F, Soave I, Lo Monte G, Patella A, Caserta D, Moscarini M. Ovarian stimulation in women with high and normal body mass index: GnRH agonist versus GnRH antagonist. Gynecol Endocrinol 2012; 28: 792-795.

50) Balen AH, Platteau P, Andersen AN, Devroey P, Sørensen P, Helmgaard L, Arce JC. The influence of body weight on response to ovulation induction with gonadotrophins in 335 women with World Health Organization group II anovulatory infertility. BJOG 2006; 113: 1195-1202.

51) Esinler I, Bozdag G, Yarali H. Impact of isolated obesity on ICSI outcome. Reprod Biomed Online 2008; 17: 583-587.

52) Dechaud H, Anahory T, Reyftmann L, Loup V, Hamamah S, Hedon B. Obesity does not adversely affect results in patients who are undergoing in vitro fertilization and embryo transfer. Eur J Obstet Gynecol Reprod Biol 2006; 127: 88-93.

53) Martinuzzi K, Ryan S, Luna M, Copperman AB. Elevated body mass index (BMI) does not adversely affect in vitro fertilization outcome in young women. J Assist Reprod Genet 2008; 25: 169-175.

54) Bonetti TC, Salomao R, Brunialti M, Braga DP, Borges E Jr, Silva ID. Cytokine and hormonal profile in serum samples of patients undergoing controlled ovarian stimulation: interleukin-1beta predicts ongoing pregnancy. Hum Reprod 2010; 25: 2101-2016.

55) Altun T, Jindal S, Greenseid K, Shu J, Pal L. Low follicular fluid IL-6 levels in IVF patients are associated with increased likelihood of clinical pregnancy. J Assist Reprod Genet 2011; 28: 245-251.

56) Mori T. Immuno-endocrinology of cyclic ovarian function. Am J Reprod Immunol 1990; 24: 80-89.

57) Orvieto R. Controlled ovarian hyperstimulation: an inflammatory state. J Soc Gynecol Investig 2004; 11: 424-426.

58) Viganò P, Fusi F, Gaffuri B, Bonzi V, Ferrari A, Vignali M. Soluble intercellular adhesion molecule-1 in ovarian follicles: production by granulosa luteal cells and levels in follicular fluid. Fertil Steril 1998; 69: 774-779.

59) Fornaro F, Cobellis L, Mele D, Tassou A, Badolati B, Sorrentino S, De Lucia D, Colacurci N. Effects of gonadotropin-releasing hormone agonist/recombinant follicle-stimulating hormone versus gonadotropin-releasing hormone antagonist/recombinant follicle-stimulating hormone on follicular fluid levels of adhesion molecules during in vitro fertilization. Fertil Steril 2007; 87: 39-47.

60) Neufeld G, Cohen T, Gengrinovitch S, Poltorak Z. Vascular endothelial growth factor (VEGF) and its receptors. FASEB J 1999; 13: 9-22.

61) Klein NA, Battaglia DE, Woodruff TK, Padmanabhan V, Giudice LC, Bremner WJ, Soules MR. Ovarian follicular concentrations of activin, follistatin, inhibin, insulin-like growth factor I (IGF-I), IGF-II, IGF-binding protein-2 (IGFBP-2), IGFBP-3, and vascular endothelial growth factor in spontaneous menstrual cycles of normal women of advanced reproductive age. J Clin Endocrinol Metab 2000; 85: 4520-4525.

62) Battaglia C, Genazzani AD, Regnani G, Primavera MR, Petraglia F, Volpe A. Perifollicular Doppler flow and follicular fluid vascular endothelial growth factor concentrations in poor responders. Fertil Steril 2000; 74: 809-812.

63) Ocal P, Aydin S, Cepni I, Idil S, Idil M, Uzun H, Benian A. Follicular fluid concentrations of vascular endothelial growth factor, inhibin A and inhibin B in IVF cycles: are they markers for ovarian response and pregnancy outcome? Eur J Obstet Gynecol Reprod Biol 2004; 115: 194-199.

64) Ferrari B, Pezzuto A, Barusi L, Coppola F. Follicular fluid vascular endothelial growth factor concentrations are increased during GnRH antagonist/FSH ovarian stimulation cycles. Eur J Obstet Gynecol Reprod Biol 2006; 124: 70-76.

65) Tarlatzis BC, Fauser BC, Kolibianakis EM, Diedrich K, Rombauts L, Devroey P. GnRH antagonists in ovarian stimulation for IVF. Hum Reprod Update 2006; 12: 333-340.

66) Weiss JM, Oltmanns K, Gürke EM, Polack S, Eick F, Felberbaum R, Diedrich K, Ortmann O. Actions of gonadotrophin-releasing hormone antagonists on steroidogenesis in human granulosa lutein cells. Eur J Endocrinol 2001; 144: 677-685.

67) Centurione L, Giampietro F, Sancilio S, Piccirilli M, Artese L, Tiboni GM, Di Pietro R. Morphometric and ultrastructural analysis of human granulosa cells after gonadotrophin-releasing hormone agonist or antagonist. Reproductive BioMedicine Online 2010; 20: 625- 633.

68) Giampietro F, Sancilio S, Tiboni GM, Rana RA, Di Pietro R. Levels of apoptosis in human granulosa cells seem to be comparable after therapy with a gonadotropin-releasing hormone agonist or antagonist. Fertil Steril 2006; 85: 412-419.

69) Gómez-Palomares JL, Acevedo-Martin B, Chavez M, Manzanares MA, Ricciarelli E, Hernandez E. Multifollicular recruitment in combination with gonadotropin-releasing hormone antagonist increased pregnancy rates in intrauterine insemination cycles. Fertil Steril 2008; 89: 620-624.

70) Gómez-Palomares JL, Julià B, Acevedo-Martìn B, Martìnez-Burgos M, Hernàndez ER, Riciarelli E. Timing ovulation for intrauterine insemination with a GnRH antagonist. Hum Reprod 2005; 20: 368-372.

71) Ragni G, Vegetti W, Baroni E, Colombo M, Arnoldi M, Lombroso G, Crosignani PG. Comparison of luteal phase profile in gonadotrophin stimulated cycles with or without a gonadotrophin-releasing hormone antagonist. Hum Reprod 2001; 16: 54-58.

72) Cantineau AE, Cohlen BJ, Heineman MJ. Ovarian stimulation protocols (anti-oestrogens, gonadotrophins with and without GnRH agonists/antagonists) for intrauterine insemination (IUI) in women with subfertility. Cochrane Database Syst Rev 2007; 18: CD005356.

73) Revelli A, Casano S, Salvagno F, Delle Piane L. Milder is better? Advantages and disadvantages of "mild" ovarian stimulation for human in vitro fertilization. Reprod Biol Endocrinol 2011; 9: 25.