0163-769X/06/$20.00/0
Printed in U.S.A.

Endocrine Reviews 27(2):170–207

doi: 10.1210/er.2005-0015

The Science behind 25 Years of Ovarian Stimulation for *in Vitro* Fertilization

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To allow selection of embryos for transfer after *in vitro* fertilization, ovarian stimulation is usually carried out with exogenous gonadotropins. To compensate for changes induced by stimulation, GnRH analog cotreatment, oral contraceptive pretreatment, late follicular phase human chorionic gonadotropin, and luteal phase progesterone supplementation are usually added. These approaches render ovarian stimulation complex and costly. The stimulation of multiple follicular development disrupts the physiology of follicular development, with consequences for the oocyte, embryo, and endometrium.

In recent years, recombinant gonadotropin preparations have become available, and novel stimulation protocols with less detrimental effects have been developed. In this article, the scientific background to current approaches to ovarian stimulation for *in vitro* fertilization is reviewed. After a brief discussion of the relevant aspect of ovarian physiology, the development, application, and consequences of ovarian stimulation strategies are reviewed in detail. (*Endocrine Reviews* 27: 170–207, 2006)

First Published Online January 24, 2006

Abbreviations: AMH, Anti-Mullerian hormone; ART, assisted reproductive technique; CC, clomiphene citrate; CG, chorionic gonadotropin; CTP, carboxy-terminal peptide; DVT, deep vein thrombosis; E_2, estradiol; EGF, epidermal growth factor; ER, estrogen receptor; FISH, fluorescent *in situ* hybridization; hCG, human CG; hMG, human menopausal gonadotropin; IVF, *in vitro* fertilization; IVM, *in vitro* maturation; LIF, leukemia inhibitory factor; OC, oral contraceptive; OHSS, ovarian hyperstimulation syndrome; P, progesterone; PCOS, polycystic ovary syndrome; PGS, preimplantation genetic screening; PR, P receptor; recFSH, recombinant FSH; rechCG, recombinant hCG; recLH, recombinant LH; uFSH, urinary FSH; uhCG, urinary hCG; VEGF, vascular endothelial growth factor; VTE, venous thromboembolism.

***Endocrine Reviews* is published by The Endocrine Society (http://www.endo-society.org), the foremost professional society serving the endocrine community.**

I. Introduction

SINCE THE PIONEERING days of *in vitro* fertilization (IVF), ovarian stimulation has been an integral part of assisted reproductive techniques (ARTs). The goal of ovarian stimulation is to induce ongoing development of multiple dominant follicles and to mature many oocytes to improve chances for conception either *in vivo* (empirical ovarian stimulation with or without intrauterine insemination) or *in vitro* (with IVF) (1, 2). This approach of interfering with physiological mechanisms underlying single dominant follicle selection is usually applied in normo-ovulatory women (3). This should be clearly differentiated from ovulation induction, which aims to induce monofollicular development and ovulation in anovulatory women (4). Ovarian stimulation enables the retrieval of many cumulus-oocyte complexes (Fig. 1). This allows for inefficiencies in subsequent oocyte maturation, fertilization *in vitro*, embryo culture, embryo

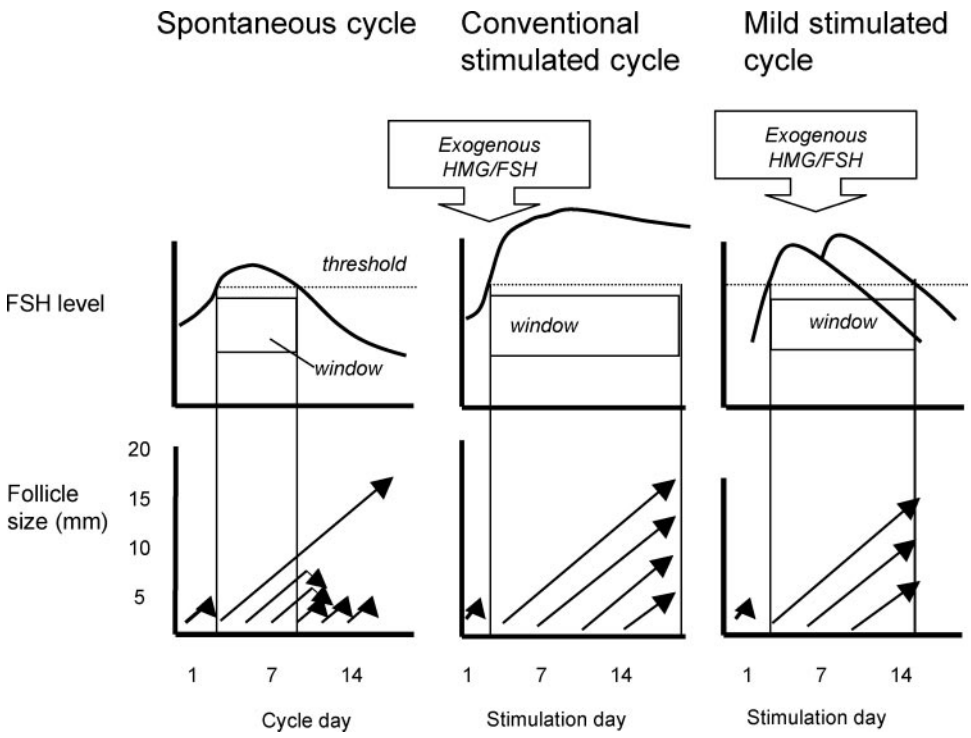

FIG. 1. The FSH threshold and window concept for monofollicular selection (*left panel*), as conventionally applied to achieve multifollicular development (*middle panel*). Each *arrow* represents a developing follicle. The *right panel* represents the concept of extending the FSH window by administering exogenous FSH in the midfollicular phase to maintain FSH levels above the threshold allowing multifollicular development to occur.

selection for transfer, and implantation (5). Multiple embryos can be transferred in the great majority of patients, and spare embryos may be cryopreserved to allow for subsequent chances of pregnancy without the need for repeated ovarian stimulation and oocyte retrieval (6, 7).

This paradigm for ovarian stimulation has formed the basis of clinical practice since the early days of IVF. In this article the scientific basis, clinical application, effects, and outcomes of current approaches to ovarian stimulation for ART will be addressed.

II. Physiology of Ovarian Function Relevant to Ovarian Stimulation

A. Endocrine control of follicular development

Initiation of growth of primordial follicles, also referred to as primary recruitment, occurs continuously and in a random fashion. Follicle development from the primordial to the preovulatory stage takes several months (8, 9). The great majority of primordial follicles that enter this development phase undergo atresia before reaching the antral follicle stage, principally through a process of apoptosis. The degree to which early stages of follicle development are influenced by FSH remains unclear. Studies in hypophysectomized and transgenic mice suggest that gonadotropins may be involved in the activation of resting follicles (10, 11). However, human FSH receptor mRNA is only expressed from the primary follicle onward. Studies in women with a mutated FSH β-subunit have shown follicular growth to occur up to the stage of secondary recruitment (12). In addition, exogenous FSH can stimulate follicle growth up to the preovulatory stage in hypophysectomized women (13). Factors such as TGF-α from theca cells, growth differentiation factor 9, and bone morphogenetic protein 15 produced by the oocyte may limit the effects of FSH on granulosa cell differentiation and follicle development at this early stage (14). Only at more advanced stages of development do follicles become responsive to FSH and obtain the capacity to convert the theca-cell derived substrate androstenedione to estradiol (E_2) by the induction of the aromatase enzyme activity (4, 15).

Due to the demise of the corpus luteum during the late luteal phase of the menstrual cycle, E_2, inhibin A, and progesterone (P) levels fall. This results in an increased frequency of pulsatile GnRH secretion, inducing rising FSH levels at the end of the luteal phase (16, 17). Although each growing follicle may initially have an equal potential to reach full maturation, only those antral follicles that happen to be at a more advanced stage of maturation during this intercycle rise in FSH (levels surpassing the so-called threshold for ovarian stimulation) gain gonadotropin dependence and continue to grow (4) (Fig. 1). This process is referred to as cyclic, gonadotropin-dependent or "secondary" recruitment, as opposed to the initial gonadotropin-independent "primary" recruitment of primordial follicles (9). Based on indirect observations, it is believed that the cohort size of healthy early antral follicles recruited during the luteo-follicular transition is around 10 per ovary (8, 18, 19)

In the subsequent follicular phase, FSH levels plateau during the initial days (20, 21) and are gradually suppressed thereafter by ovarian inhibin B (22) and E_2 (23) negative feedback. Gonadotropin withdrawal studies have demonstrated the association between FSH, LH, and inhibin production (16, 24, 25). Administration of recombinant inhibin A during the early follicular phase to nonhuman primates results in a gradual decrease in FSH (26). On the contrary, administration of recombinant inhibin A during the luteal phase prevents the subsequent rise of FSH during the luteo-

follicular transition (26). These experiments suggest a direct endocrine role for inhibin A in the negative feedback on pituitary FSH production, whereas inhibin B does not contribute to the dynamic changes within a menstrual cycle (26–29).

Decremental follicular phase FSH levels (effectively restricting the time where FSH levels remain above the threshold, referred to as the FSH window) (Fig. 1) appear to be crucial for selection of a single dominant follicle from the recruited cohort (20). As FSH levels fall, all but the dominant follicle (with its increased sensitivity to FSH) lose the stimulus to further development and become atretic (4, 30). The important concept of increased sensitivity of the dominant follicle for FSH has been confirmed by human studies showing developing follicles to exhibit variable tolerance to GnRH antagonist-induced gonadotropin withdrawal (31, 32). Recent evidence also points to a central role for LH in monofollicular selection and dominance in the normal ovulatory cycle (33, 34). Although granulosa cells from early antral follicles respond only to FSH, those from mature follicles (exhibiting receptors to both gonadotropins) are responsive to both FSH and LH. The maturing dominant follicle may become less dependent on FSH because of the ability to respond to LH (33–35).

Cyclic variation in the expressed isoforms of FSH (differing in oligosaccharide structure, the degree of terminal sialylation and sulfation) has been described (36). A greater proportion of less acidic circulating FSH isoforms are observed during the late follicular phase and midcycle (37–39). The half-life of human FSH secreted 2–3 d before ovulation is considerably shorter than during the early follicular phase (40). It has been suggested that the preferential secretion of short-lived isoforms during the periovulatory period indicates the existence of regulatory mechanisms that control the intensity and duration of the FSH signal delivered to the ovary (36).

B. Intraovarian modulators of steroidogenesis

Gonadotropins are the primary regulators of follicular development, cytodifferentation, and sex-steroid production in the ovary. However, a large number of intraovarian regulators modulate the response to gonadotropin stimulation. The principal regulatory systems in the human involve the IGF system (41), the epidermal growth factor (EGF) system (42), and the TGF-α and -β systems (43). In addition to their primary endocrine and paracrine functions of suppressing FSH secretion by the pituitary (44), inhibins and activins also exhibit local actions in the ovary (45).

Although IGFs and their receptors are known to be present in developing human follicles, uncertainty remains regarding the individual role of the different IGFs, their receptors, and binding proteins *in vivo*. *In vitro* studies have identified the effects of IGFs on granulosa and theca cell function. IGF-I has been shown to stimulate proliferation and aromatase activity of granulosa cells both alone or synergistically with FSH (46–49). At the theca cell, IGF-I stimulates production of 17 α-hydroxyprogesterone (50), whereas both IGF-I and -II can alone or together with LH stimulate androgen synthesis (51, 52). For review, see Poretsky *et al*. (41).

The activity of the IGFs is modulated by their degree of binding to IGF binding proteins. GH is the primary regulator of serum IGF-levels (41, 53). However, GH does not affect IGF-I and IGF-II expression in granulosa cells *in vitro*. It has therefore been proposed that GH may indirectly modulate the follicle by stimulating hepatic production of IGF-I.

EGF and TGF-α are structurally similar polypeptides that bind to a common receptor, and both have been detected in human follicles (54). They would appear to stimulate granulosa cell proliferation (42) but inhibit FSH-induced aromatase expression and E_2 synthesis (55). TGF-β differs in structure and function from EGF and TGF-α, being a homodimeric polypeptide with no clear direct inhibitory function on granulosa cell aromatase activity. Both EGF and TGF-β synergize with FSH to stimulate granulosa cell proliferation in hamster preantral follicles (56).

In addition to regulating pituitary FSH, inhibin and activin also act as paracrine and autocrine modulators of ovarian follicle growth and maturation (57).

Activin acts on small follicles to stimulate proliferation of granulosa cells (58, 59), up-regulates FSH receptor expression in granulosa cells, and increases aromatase expression, resulting in increased E_2 production (60). Inhibin augments LH-stimulated androgen production by thecal cell cultures (61).

C. Control of corpus luteum function

As reviewed recently (62, 63), it is generally believed that the predominant hormonal regulators of the corpus luteum in women and many nonhuman primates are LH-like gonadotropins. Unlike in some species, notably rodents (64), the luteotropic process in humans does not include a principal role for prolactin-like hormones, and luteal regression does not involve a uterine signal (prostaglandin $F_{2\alpha}$) (63). Instead, it is: 1) the midcycle surge of gonadotropins (notably, LH) secreted by the anterior pituitary that stimulates resumption of meiosis and oocyte maturation in the preovulatory follicle, rupture of the ovulatory follicle and release of the expanded cumulus-oocyte complex, and conversion of the follicle wall into the corpus luteum (*i.e.* luteinization); 2) the pulsatile secretion of pituitary LH during the luteal phase of the menstrual cycle that promotes the continued development and normal functional lifespan of the corpus luteum; 3) the exponential rise in circulating levels of the LH-like hormone, chorionic gonadotropin (CG), secreted by the implanting blastocyst and syncytiotrophoblast of the developing placenta, that extends the functional lifespan of the corpus luteum in early pregnancy until luteal activities are assumed by the placenta, *i.e.*, at the luteal-placental shift.

Elegant studies during the 1970s and 1980s (for reviews, see Refs. 65 and 66) using techniques such as gonadotropin ablation and GnRH infusion to control LH secretion established the critical role of LH/CG in regulating primate luteal structure-function. More recent experiments using GnRH antagonists and pure recombinant human LH or human CG (hCG) have strengthened this concept (67–70) and clarified a number of issues.

First, although the maturing dominant follicle may be less sensitive to acute LH withdrawal at midcycle, a GnRH-in-

duced LH surge of substantial length is required for ovulation and development of normal luteal function. Of considerable interest is how the duration and/or amplitude of the midcycle LH surge influences periovulatory events. Although more research is needed, initial monkey and human studies on GnRH-induced LH surges or administering exogenous LH/CG suggest that surges of lesser duration (<24 h) and amplitude are sufficient to reinitiate oocyte meiosis and granulosa cell luteinization, but surges of greater duration (>24 h) and amplitude improve oocyte recovery, fertilization, and corpus luteum development (71, 72). Although the LH surge is believed to be the physiological signal for periovulatory events, studies in rodents (73) showed that a midcycle bolus of FSH can replace LH and elicit oocyte maturation, ovulation, and successful pregnancy. Likewise, an FSH bolus will induce certain periovulatory events in macaque follicles (74) after ovarian stimulation, including oocyte meiotic maturation, fertilization, and early luteinization of granulosa cells.

Secondly, although luteinizing tissue or cells appear less responsive to exogenous gonadotropin around the time of ovulation (presumably due to LH/CG receptor desensitization, down-regulation, or occupancy by gonadotropins from the surge interval), the developing corpus luteum in the early luteal phase (70) is comparable to the developed corpus luteum by midluteal phase (32) in its critical dependence on circulating LH for continued function. Several reports confirm that suppression of LH support for 72 h results in irreversible loss of luteal structure-function (69, 70), but LH or CG (not FSH) replacement sustains luteal structure-function (75, 76). Attempts to titrate the amount of LH required to maintain the normal functional lifespan of the corpus luteum in GnRH antagonist-treated monkeys showed that increasing the dose from mid-to-late luteal phase was critical (77, 78). Such results support the concept that the primate corpus luteum becomes less sensitive to LH as the luteal lifespan progresses. Although the frequency of LH pulses declines during the luteal phase (79), prevention of this phenomenon (via pulsatile GnRH infusion or LH injections (80) does not prevent timely luteal regression. Rather, decreasing luteal sensitivity to gonadotropin could be a critical factor in timely luteolysis near the end of the menstrual cycle (66).

Collectively, the data suggest that rescue of the corpus luteum from impending regression and continuation of its functional lifespan in early pregnancy are not likely due to inherent differences in LH *vs.* CG bioactivity or to a change from pulsatile (LH) to continuous (CG) gonadotropin exposure in the fertile cycle. Rather, it appears that a more robust luteotropic stimulus, in the form of rising levels of LH/CG, is required to extend the functional lifespan of the primate corpus luteum.

Local modulating factors may include the steroid hormones produced by the corpus luteum. There is considerable evidence that another action of the midcycle LH surge, which complements the promotion of P production, is the induction of P receptors (PRs) in luteinizing granulosa cells of the follicle (for review, see Ref. 62). The hypothesis that estrogen acts locally as a luteolytic signal (81) has renewed credence with the discovery of estrogen receptor (ER)-β in the primate corpus luteum (82). Although there are reports of androgen receptors in primate luteal tissue (83, 84), there has been little consideration of local androgen action in the corpus luteum.

LH/CG also regulates the expression of angiogenic and angiolytic factors that likely control the expanding vasculature in the ovulatory follicle and developing corpus luteum. The LH-stimulated luteinization of granulosa cells around ovulation includes enhanced vascular endothelial growth factor (VEGF) production (85), which is likely essential for the angiogenic process within the primate corpus luteum (86, 87). With respect to hCG, a midcycle bolus in ovarian stimulation cycles increased expression of the endogenous angiopoietin agonist, Ang-1, without altering that of the endogenous antagonist, Ang-2 (88), in macaque granulosa cells. It is important to recognize that these factors control not only the development or maintenance of the vasculature in developing tissue beds, but also vascular integrity, maturity, and permeability. It has been proposed (89, 90) that overexpression, increased bioavailability, or a change in the ratio of angiogenic factors, notably VEGF-A, is a cause of ovarian hyperstimulation syndrome (OHSS) (91), a serious side effect of ovarian stimulation characterized by intravascular volume loss and extravascular fluid accumulation. The early or late occurrence of OHSS in ovarian stimulation cycles has been linked to the ovulatory hCG bolus and endogenous CG production at pregnancy recognition, respectively.

D. Control of endometrial function

1. Steroid hormone receptors and actions in endometrium. Ovarian-derived steroid hormones have profound effects on the endometrium that result in proliferation and differentiation of the tissue, receptivity to embryonic implantation, and shedding in the absence of a pregnancy. During the follicular phase, E_2 secreted by growing follicles stimulates ER expression, with highest levels observed in glandular epithelium during the late follicular phase (92–97). Two forms of ER are now appreciated: ER-α and ER-β, which are two distinct gene products (98). They are expressed in both glands and stroma (with ER-α predominating), whereas ER-β is only expressed in endothelium (99). ER(α)is significantly down-regulated in epithelium in the luteal phase, a universal response in all mammalian species (100).

With regard to PR, peak expression in human endometrium induced by E_2 is observed at the time of ovulation (92, 97, 101, 102). PR is most prominent in glandular epithelium in the proliferative phase and is undetectable in the midluteal phase in this cell type (92). In contrast, stromal cells have high levels of PR in the follicular phase and throughout the luteal phase. Similar observations have been made in nonhuman primate endometrium (93, 95, 96, 103). The human PR has two functionally distinct isoforms, PR-A and PR-B, encoded by a single gene (104). In endometrial glands, PR-A and PR-B are expressed in the follicular phase, but only PR-B persists during the mid- and late luteal phase in this cell type (102, 105, 106). In endometrial stroma, PR-A predominates throughout the cycle, suggesting that it is important in P action in the luteal phase (102, 105, 106). Overall, these results support the view that PR-A and PR-B mediate distinct pathways of P action in the glandular epithelium and stroma throughout the menstrual cycle. It should be noted that the

timely down-regulation of epithelial PR coincides with the opening of the window of implantation and uterine receptivity for embryonic implantation (see *Section II.D.3*), and histological delay of the endometrium (a clinically abnormal state) is associated with a failure of such PR down-regulation (107).

The major roles of E_2 are for endometrial growth and for enabling P to act on the tissue. To accomplish these goals, E_2 induces PR expression and promotes cellular proliferation in the tissue—directly through its cognate receptors, and indirectly by induction of growth factors that act as autocrine and/or paracrine modulators (108–110).

2. Endometrial morphological changes in response to ovarian-derived steroid hormones. The endometrium demonstrates day-by-day morphological changes, extensively described by Noyes *et al*. (111) who analyzed several thousand endometrial biopsies to develop the criteria for assessment that are still considered the gold standard. The initial Noyes' criteria correlated the results of the biopsy with the basal body temperature and with the subsequent menstrual period. Extensive ultrastructural changes also occur during the cycle that underscore the magnitude of effects of ovarian-derived steroid hormones on this tissue (112).

Morphological features correlating with endometrial maturity have been identified by scanning electron microscopy. Pedunculated extrusions of the luminal epithelial cell membrane, termed pinopodes, can be identified near the lateral cell border, rising above the plane of the normal microvilli, but not occupying the entire cell surface (113). They are P-dependent and inhibited by E_2 (114–116). These structures last for 1–2 d, and their numbers positively correlate with implantation sites (117). Although they likely play a role in the early stages of implantation, their precise functions remain to be clarified.

3. The window of implantation and the effects of ovarian-derived steroid hormones. Ovarian steroid hormone actions during normal ovulatory and hyperstimulation cycles result in a temporally and spatially restricted period ("window of implantation") in which the tissue is receptive to embryonic implantation (118). Available evidence supports the discrete time in the cycle between 6 and 10 d after the LH surge that defines the window of implantation. The window is advanced in clomiphene citrate (CC) or gonadotropin-stimulated cycles (114, 119) and is delayed in steroid hormone replacement cycles for donor recipients (120), underscoring its plasticity and the significant effects that steroid hormones have on this tissue. When embryo transfers were performed in IVF cycles between cycle d 17 and 19, 40.5% conceptions occurred, compared with no conceptions in cycles where embryo transfers occurred after cycle d 20 (121). These observations collectively support a distinct and narrow period of endometrial specialization that coincides with the window of implantation.

A major challenge is to define the molecular events occurring during the window of implantation that render the endometrium receptive to implantation and the interactions that occur between the maternal endometrium during pregnancy (*i.e.*, the decidua) and the implanting conceptus. Immunohistochemical techniques have characterized the expression of receptors, adhesion molecules, and other markers of receptivity. The presence of ER and PR is most pronounced during ovulation (122). These nuclear receptors are especially induced by ovarian estrogens and are present in the glandular and stromal compartment (92).

The expression of cell adhesion molecules such as integrins is also under endocrine and paracrine control (92). Flow cytometry studies have shown E_2 and P to decrease $\alpha V\beta 3$ integrin expression. Down-regulation of this integrin by E_2 and P indicates that implantation and receptivity may arise as a result of a down-regulation of E_2 and PRs during the midluteal phase (92, 123). E_2 and P may therefore have a suppressive role on integrins and other critical endometrial proteins such as cytokines, which may only be expressed when this inhibitory signal is removed.

Leukemia inhibitory factor (LIF) is the first cytokine that appeared to be critically involved in embryonic development and implantation in mice. LIF is a secreted pleiotropic cytokine with a glycoprotein structure of 180 amino acids (33). High serum P levels coincide with the presence of LIF, and glandular LIF expression can be blocked by antiprogestins (124). The biological action of LIF in human endometrium is still unclear, but it probably has a function in human implantation at the stage of embryonic invasion. Coexpression of LIF and pinopodes has been found in fertile women (125).

Functional genomic approaches have been used to defined the molecular events occurring during the implantation window that contribute to the interactions between the endometrium and an implanting conceptus (126–129). Moreover, new light has been shed on the impact of ovarian hyperstimulation on endometrial gene expression. Putative molecular players in the endometrium for uterine receptivity and their roles in the early stages of implantation have been reviewed in the mouse (130), the nonhuman primate (131), and the human (118, 131–133).

E. Ovarian aging

By a process of mitosis, the pool of germ cells undergoes rapid expansion, reaching a maximum of approximately 7×10^6 oogonia by the fifth month of intrauterine life (134). The oogonia then enter meiotic prophase, marking the completion of germ cell production. From here on, attrition in germ cell numbers occurs such that at birth each ovary contains between 25×10^4 and 50×10^4 resting follicles (135, 136). Depletion of these primordial follicles, already begun before birth, continues throughout childhood so that by the menarche a total of approximately 3×10^4 remain (137). During reproductive life, follicle depletion occurs at a rate of approximately 1000 per month by either atresia or entry into the growth phase, and this rate increases after the age of 35 yr (138) until the menopause when the stock of resting follicles falls to less than 1000 per ovary (135, 138).

The vast majority of follicles are removed from this stock by apoptosis (139). During fetal life and childhood, follicle development occurs up to the early antral stage (140). From puberty until the menopause, full maturation and ovulation occur, but only approximately 400 follicles are destined to achieve full maturation. As reproductive age advances to the menopause, the menstrual cycle decreases in length predom-

inantly due to a shortening of the follicular phase (141). The shortened follicular phase in older ovulatory women is due to advanced follicle growth and earlier dominant follicle selection (142). Depletion of the ovarian follicular pool (138) leads to a diminished production of E_2 and inhibin-B (143) and a gradual rise in FSH concentrations (144). Major individual variability exists in the rate of follicle pool depletion within the normal range of menopausal age of 40 to 60 yr (145) (Fig. 2). Hence, chronological age is only loosely associated with the extent of follicle depletion (ovarian age).

The long-held paradigm of continued depletion of the fixed number of oocytes laid down during fetal life has recently been questioned. Female mice appear to contain a population of germline stem cells that may maintain follicle numbers during adult life (146). Recently, expression of germline markers in bone marrow has been demonstrated in mice, implicating bone marrow as a potential source of germ cells (147). If confirmed by other investigators, these findings are likely to have an impact on concepts of ovarian aging and the development of therapeutic interventions designed to maintain ovarian reserve in the human.

The identification of sensitive and specific markers of ovarian aging may enable prediction of individual response to ovarian stimulation and outcome of IVF. The most widely used endocrine marker for ovarian reserve is the early follicular phase FSH level (144). Although baseline FSH levels predict poor response to ovarian stimulation (148, 149), age appears to be more closely related to the chance of implantation and ongoing pregnancy (150, 151). Young women with high FSH levels demonstrate lower numbers of growing follicles but can achieve good ongoing pregnancy rates if oocytes and embryos are obtained (152, 153). Inconsistencies may arise from the wide interindividual variation that exists in follicular phase FSH concentrations in the normo-ovulatory cycle (21, 154) and from discrepancies between quantity (follicle number) and quality (competence) of oocytes.

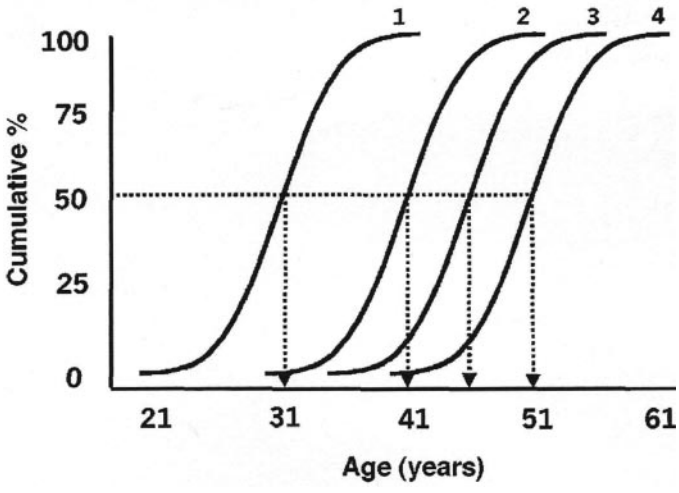

FIG. 2. The age variations of the various stages of reproductive aging given by four curves representing 1) age at the beginning of subfertility, 2) age at beginning of sterility, 3) age at transition from cycle regularity to irregularity, and 4) age at menopause. The *dotted lines* indicate the age at which 50% of the female population has reached each given stage of reproductive aging. [Reproduced with permission from E. R. te Velde and P. L. Pearson: *Hum Reprod Update* 8:141–154, 2002 (145). © The European Society of Human Reproduction and Embryology. Reproduced by permission of Oxford University Press/ Human Reproduction.]

Elevated E_2 levels on cycle d 3 may also predict poor response to ovarian stimulation for ART, even when baseline FSH levels are normal (155). Because FSH levels are not always correlated with E_2 concentrations, the rising FSH levels are partly attributed to lower inhibin levels produced by the aging ovary (143). A decrease in serum inhibin B precedes both the fall in inhibin A levels (156) and the perimenopausal rise in basal FSH (157). Falling inhibin B concentrations may also provide a more direct assessment of ovarian reserve, because it is directly produced by developing early antral follicles (22, 158). Studies concerning the value of assessing inhibin B have shown discordant results (22, 157, 159–162). Recently, it was demonstrated in a multivariate analysis that addition of basal FSH and inhibin B levels to a logistic model including ultrasound characteristics can improve the performance of the prediction model for ovarian response to stimulation (163).

The age-related decrease in number of antral follicles present in the ovary at the start of the cycle is considered to correlate with the number of primordial follicles remaining in the ovary (164). It should be emphasized, however, that direct evidence to support this contention is lacking. The antral follicle number assessed by ultrasound during the early follicular phase has been shown to correlate with ovarian response to stimulation (163, 165), and to predict the number of immature oocytes retrieved from unstimulated ovaries before *in vitro* maturation (IVM) (166). Ovarian volume, which partly reflects the number of ovarian follicles, has also been shown to decrease with age (167), and a number of studies have suggested a role for this parameter as a marker of ovarian reserve (168–170).

Recently, anti-Mullerian hormone (AMH), also referred to as Mullerian-inhibiting substance, has also been studied as a marker of ovarian aging. A member of the TGF-β superfamily, AMH is produced by ovarian granulosa cells from about 36 wk gestation to the menopause (171). Expression of AMH is highest in granulosa cells of growing preantral and small antral follicles. The role of AMH in follicle development and function has been elucidated in studies of AMH-deficient mice. In the absence of AMH, ovaries are more quickly depleted of primordial follicles due to increased recruitment (172). Additional studies have suggested that AMH may also influence the sensitivity of growing follicles to FSH (173). *In vitro* studies have shown AMH to reduce expression of aromatase mRNA, and decrease the number of LH receptors in granulosa cells (174). *In vivo* studies in AMH null mice have supported an inhibitory role for AMH in the cyclic recruitment of follicles by lowering sensitivity to FSH (175). Because AMH is produced by growing follicles, it has been proposed as a marker of ovarian reserve. Indeed, serum concentrations of AMH decrease over time in young normo-ovulatory women (176). AMH concentrations correlate well with the number of antral follicles and age, and less strongly with inhibin B and FSH levels (176, 177). In contrast to inhibin B and FSH, serum AMH levels are relatively constant throughout the menstrual cycle. Taken together with recent clinical studies showing high correlations between low AMH levels

and ovarian response to stimulation (178, 179), AMH may represent an important marker of ovarian reserve.

III. The Development of Ovarian Stimulation Agents

A. Background

Evidence of the endocrine pituitary-gonadal axis arose early in the 20th century when it was observed that lesions of the anterior pituitary resulted in atrophy of the genitals. The first convincing evidence supporting the existence of two separate gonadotropins (initially referred to as Prolan A and Prolan B) was provided by Fevold *et al*. in 1931 (180), and both LH and FSH were subsequently isolated and purified. In 1928, Ascheim and Zondek (181) described the capacity of urine from pregnant women to stimulate gonadal function. The concept of stimulating ovarian function by the exogenous administration of gonadotropin preparations has intrigued investigators for many decades. In 1940, Hamblen (182) reported the ability of purified pregnant mare serum to induce ovulation in humans by iv administration. However, these early attempts had to be stopped due to species differences and resulting antibody formation impacting on efficacy and safety. Clinical experiments in the late 1950s demonstrated that extracts derived from the human pituitary could be used to stimulate gonadal function (183). Subsequently, experiments involving the extraction of both the gonadotropic hormones LH and FSH from urine of postmenopausal women led to the development of human menopausal gonadotropin (hMG) preparations. From the early 1960s, these were used for the stimulation of gonadal function in the human (for historic overview, see Ref. 184). A second important development allowing for ovarian stimulation on a large scale arose when the first estrogen antagonist tested in cancer patients was found to induce ovulation.

B. The discovery of clomiphene citrate

In the late 1950s, the first nonsteroidal estrogen antagonist (MER-25) was tested for the treatment of breast cancer and endometrial hyperplasia. The administration of CC in women with endometrial hyperplasia suffering from secondary amenorrhea was followed by the recommencement of menstrual cycles (185). Shortly thereafter, the ovulation-inducing capacity of a closely related antiestrogen (MRL/41) was recognized (186). CC was originally developed for clinical use by the Merrel company in 1956. Nearly 50 yr later, it is still considered to represent the first line treatment strategy in most anovulatory infertility and is still the most applied drug for infertility therapies worldwide.

CC is an oral antiestrogen consisting of a racemic mixture of two stereoisomeres. The enclomiphene isomere has a relatively short half-life, whereas the zuclomiphene isomere has an extended clearance and may accumulate over consecutive cycles. The two isomers demonstrate different patterns of agonistic and antagonistic activity *in vitro* (187, 188). Stimulation of ovarian function is elicited by raised pituitary FSH secretion due to blockage of E_2 steroid feedback by CC. Overall, a 50–60% increase of serum FSH levels above baseline has been described (189–191). The exact nature of the mechanism of action of CC is still uncertain (189, 192), but induced changes in the IGF system may also be important (191). CC for ovulation induction in anovulatory women is considered to be relatively safe because steroid negative feedback remains intact. The oral route of administration and low costs represent additional advantages of this preparation.

In addition to its desirable central action of stimulating a transient increase in gonadotropin secretion, CC may have other potentially detrimental effects on peripheral reproductive functions. *In vitro* studies have revealed inhibition of human granulosa or luteal cell steroidogenesis (188). However, in the context of higher E_2 levels as a result of dominant follicle growth, this is probably not of clinical importance. Antiestrogenic effects at the uterine level (cervical mucus production and endometrial receptivity) are believed to underlie the observed discrepancy between achieved ovulation and pregnancy rates (193, 194). CC does not appear to be associated with preterm birth and congenital abnormalities (195, 196). However, data from well-designed prospective studies are lacking. *In vitro* animal studies only reveal effects on oocytes or embryos when exposed to levels far higher than those attained *in vivo*. The putative increased risk of ovarian cancer reported to be associated with the use of CC for more than 12 months (197) has led CC to be licensed for just 6 months of use in some countries.

After the first IVF baby born in a natural cycle (198), four normal IVF pregnancies were subsequently reported after ovarian stimulation with CC (199). In the following years, many groups reported IVF results after CC, with or without gonadotropin cotreatment (200, 201).

C. Gonadotropins

Human menopausal gonadotropins first became widely used for IVF in the United States (202, 203). For over two decades, gonadotropin preparations have also been extensively applied for ovarian stimulation in ovulatory women for empirical treatment of unexplained subfertility. The aim is to increase the number of oocytes available for fertilization *in vivo* (for review, see Ref. 204).

The FSH to LH bioactivity ratio of registered hMG preparations is 1:1. As purity improved, it was necessary to add hCG to maintain this ratio of bioactivity (205). The initial preparations were very impure with many contaminating proteins; less than 5% of the proteins present were bioactive. Bioactivity of gonadotropin preparations continues to be assessed by the crude *in vivo* rat ovarian weight gain Steehlman and Pohley assay (206). This rather anachronistic technique has the disadvantage of allowing considerable batch to batch inconsistency in bioactivity. However, improved protein purification technology allowed for the production of hMG with reduced amounts of contaminating nonactive proteins and eventually the development of purified urinary FSH (uFSH) preparations by using monoclonal antibodies since the early 1980s (Fig. 3) (for review, see Ref. 184). The currently available pure products allow for less hypersensitive reactions and less painful sc administration. Due to the worldwide increased need for gonadotropin preparations, demands for postmenopausal urine increased tremendously, and adequate supplies could no longer be guaranteed. In

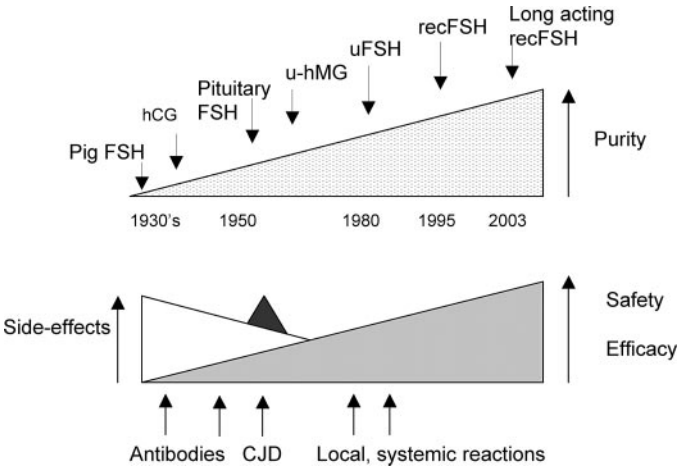

FIG. 3. Graphic overview of the main milestones in the development of gonadotropins for clinical use. [Adapted from B. Lunenfeld: *Hum Reprod Update* 10:453–467, 2004 (184). © The European Society of Human Reproduction and Embryology. Reproduced by permission of Oxford University Press/Human Reproduction.] CJD, Creutzfeldt Jakob disease.

addition, concern regarding the limited batch-to-batch consistency along with possibilities of urine contaminants emerged (207).

Through recombinant DNA technology and the transfection of human genes encoding for the common α- and hormone-specific β-subunit of the glycoprotein hormone into Chinese hamster ovary cell lines (208), the large scale *in vitro* production of human recombinant FSH (recFSH) has been realized (209, 210). The first pregnancies using this novel preparation in ovulation induction (211) and in IVF (212, 213) were reported in 1992. Since then, numerous, large-scale, multicenter studies have been undertaken (214) demonstrating their efficacy and safety. The recombinant products offer improved purity, consistency, and large-scale availability. Because of its purity, recFSH can now be administered by protein weight rather than bioactivity, and so-called "filled-by-mass" preparations (215) are now available for clinical use. During recent years, recombinant LH (recLH) (216, 217) and hCG (rechCG) (218, 219) have also been introduced for clinical application.

The first report on the design of a long-acting FSH agonist was by Boime and co-workers (220), who used site-directed mutagenesis and gene transfer techniques to manufacture FSH-carboxy-terminal peptide (CTP). This molecule is also produced by a Chinese hamster ovary cell line and contains four N-linked carbohydrate chains (α52, α78, β7, and β24) and four O-linked carbohydrate chains at the CTP (β115, β121, β126, and β32). The latter group causes a 3- to 4-fold increase in half-life *in vivo* compared with wild-type recFSH (221). FSH-CTP has recently been subject to clinical studies as discussed in *Section IV.B*.

D. GnRH agonists

In 1971, the small decapeptide GnRH was isolated, and its structure was elucidated (222, 223). Amino acid substitutions have revealed the significance of specific regions for its stability, receptor binding, and activation of the pituitary gonadotroph cells (224). This decapeptide is secreted by the hypothalamus into the portal circulation in an intermittent fashion stimulating the pituitary gonadotropes to synthesize and secrete LH and FSH (for review, see Ref. 225). In addition to this long-established central role, recent studies suggested that GnRH also acts as a local autocrine and/or paracrine factor in the human ovary by regulating steroidogenesis (226), cell proliferation (227), and apoptosis (228). However, the current therapeutic applications of GnRH analogs are derived from their proven role in regulating gonadotropin secretion.

Clinically safe GnRH agonists were developed by replacing one or two amino acids. An increased potency could be achieved by replacing glycine for D-amino acids at position 6 and by replacing Gly-NH_2 at position 10 by ethylamide (229). Such simple structural changes render these compounds more hydrophobic and more resistant to enzymatic degradation. In 1978, it was discovered that repeated administration of GnRH agonists produced a transient increase in gonadal function followed by a decrease in gonadal function and a significant fall in sex steroids (230, 231). Although initial binding to GnRH receptors results in activation, continuous occupation leads to desensitization due to the clustering and internalization of pituitary GnRH receptors, resulting in falling LH and FSH levels (232). If the agonists are administered for a period of several months, LH levels remain suppressed, but FSH levels return to normal and eventually rise to supraphysiological levels (233).

Pulsatile administration of GnRH was established as an effective and safe means of treating hypogonadotropic hypogonadal anovulation (231, 234). The first reports concerning its use for the prevention of a premature LH rise during ovarian stimulation appeared in the early 1980s (235, 236). During initial studies with hMG stimulation of multiple follicle development for IVF, it became apparent that a premature LH peak occurred in 20–25% of cycles due to positive feedback activity by high serum E_2 levels during the midfollicular phase of the stimulation cycle (237). This advanced exposure to high LH was associated with premature luteinization of follicles and either cycle cancellation due to follicle maturation arrest or severely compromised IVF outcomes. The clinical development of GnRH agonists (for reviews, see Refs. 225 and 229) allowed for the complete suppression of pituitary gonadotropin release during ovarian stimulation protocols for IVF (235, 238–240). Induced pituitary down-regulation indeed resulted in significantly reduced cancellation rates and improved overall IVF outcome (241). Moreover, the approach of GnRH agonist cotreatment facilitated scheduling of IVF and timing of oocyte retrieval.

Recently, a second form of GnRH agonist (GnRH-II) has been identified. This differs from the mammalian GnRH-I by three amino acid residues. In addition to expression in the brain, GnRH-I and GnRH-II transcripts are expressed in various cells within the ovary (242). The physiological significance of GnRH-II in the human remains unclear.

E. GnRH antagonists

Immediate suppression and recovery of pituitary function rendered GnRH antagonists particularly appropriate for

short-term use in IVF. However, it has taken almost three decades to develop such compounds with acceptable safety and pharmacokinetic characteristics. The first generation antagonists were developed by replacing "his" at position 2 and "trp" at position 3, but these compounds suffered from low potency. In second-generation compounds, the activity was increased by incorporating a D-amino acid at position 6. However, the widespread clinical application of these compounds was hampered by frequent anaphylactic responses due to histamine release. By introducing further replacements at position 10, third generation compounds were developed (225). Subsequently, both the compounds ganirelix (developed by Syntex Research, Palo Alto, CA) and Cetrotide (developed by Asta Medica, Frankfurt, Germany) were shown to be safe and efficacious in IVF. These third-generation GnRH antagonists were registered in 2001 for use in IVF (243).

The expression of GnRH and GnRH receptors in developing mouse embryos at the mRNA and protein levels raises issues of safety for the embryo. In one study, the incubation of mouse embryos with a GnRH agonist enhanced the preimplantation embryonic development in a dose-dependent way, whereas GnRH antagonist blocked this development (244). Moreover, GnRH mRNA and GnRH proteins are produced in the human fallopian tube during the luteal phase of the menstrual cycle (245). Studies are still required to demonstrate convincingly clinically relevant direct effects of GnRH analogs on fertilization, early embryonic development, and implantation in humans. Follow-up data on pregnancy, birth, and neonatal outcome of 227 children born after IVF or intracytoplasmic sperm injection cycles in which cetrorelix was used showed no abnormal results in comparison to outcome after commonly used long GnRH agonist protocols (246).

IV. Ovarian Stimulation Regimens

A. Clomiphene citrate

Before the introduction of GnRH agonists to induce pituitary down-regulation, combined CC/hMG regimens were considered the standard of care. The advantages of these combined regimens included reduced requirements for hMG and higher luteal phase P levels, alleviating the need for luteal phase supplementation (247). Randomized trials have been published, comparing CC stimulation with either natural cycle IVF (248) or conventional gonadotropin/GnRH agonist protocols (249). Recent studies also reported clinical outcomes of combined regimens applying CC, gonadotropins, and GnRH antagonist (250–252).

CC usually induces the development of at least two follicles, which may sometimes elicit a premature LH rise. By virtue of the fact that CC is therapeutically active through interference with estrogen feedback (requiring an intact pituitary-ovarian axis), this compound cannot be combined with GnRH agonist cotreatment for prevention of a premature LH surge. Moreover, undesired antiestrogenic effects of CC at the level of the endometrium have been implicated by some in the observed discrepancy between relatively low embryo implantation rates coinciding with successful ovarian stimulation (204). CC administration is usually initiated on cycle d 2, 3, or 5, and given daily for 5 subsequent days, with doses varying between 100 and 150 mg/d. In most applied regimens, exogenous gonadotropin medication (150 IU/d) is initiated after cessation of CC. It seems that CC alone induces a limited but dose-dependent increase in the number of developing follicles. However, the addition of gonadotropins elicits increased ovarian response as manifest by more follicles. Sufficiently powered randomized comparative trials to support one approach over the other are lacking.

Reported outcomes with CC alone are variable, but in general pregnancy rates appear higher compared with natural cycle IVF, but lower compared with conventional gonadotropin/GnRH agonist protocols. Again, most studies are uncontrolled, but an extensive summary of almost 40,000 cycles reported in the literature suggests pregnancy rates of 6% per started cycle and up to 20% per embryo transfer (253). Apart from hot flushes, which may occur in up to 10% of women taking CC, side effects are rare. Nausea, vomiting, mild skin reactions, breast tenderness, dizziness, and reversible hair loss have been reported, but less than 2% of women are affected. The mydriatic action of CC may cause reversible blurred vision in a similar number of women. Overall side effects are CC dose related and are completely reversible once medication is stopped.

Tamoxifen, like CC, is a nonsteroidal selective ER modulator. Primarily developed for and used in the treatment of breast cancer, it has also been used in ovulation induction for many years. In contrast to CC, tamoxifen only contains the zu-isomer and appears to be less antiestrogenic at the uterine level. The possible advantages of tamoxifen over CC include beneficial effects on cervical mucus (254) and an agonistic effect at the endometrium. However, although endometrial thickness may increase on ultrasound monitoring, histological studies indicate that this may be due to edema and enlargement of stromal cells, rather than a purely estrogenic proliferative effect (for review, see Ref. 255). In recent years, tamoxifen has been proposed as an alternative means of ovarian stimulation for ART in women who have had breast cancer (256, 257), while protecting the breasts from concomitant high serum estrogen levels. Additional follow-up studies should be carried out before this drug is widely applied in these patients.

B. Gonadotropins

Gonadotropin preparations still constitute the principal agents for ovarian stimulation in IVF. The daily administration of these preparations is usually efficacious in the maintenance of growth of multiple antral follicles, allowing for the retrieval of many oocytes for IVF. Preparations initially used were hMG (containing both LH and FSH bioactivity), followed by purified uFSH and more recently recFSH and recLH.

Starting doses vary between 100 and 300 IU/d and are often adjusted depending on the observed individual ovarian response. However, there is little evidence to support dose adjustments midcycle (258). Several randomized clinical trials employing GnRH agonist cotreatment have failed to demonstrate improvements in outcome when higher

doses of FSH are employed, even in older patients (259–263). A single-center study comparing 150–225 IU recFSH with GnRH antagonist comedication (264) showed similar results. The widely applied practice of increasing gonadotropin to ameliorate low response to stimulation is not supported by published evidence (265). This is not surprising when the pathophysiology of ovarian aging (*i.e.*, follicle pool depletion) is taken into consideration.

When excessive follicle development raises the concern of imminent OHSS, gonadotropins are often reduced or temporarily withheld, a practice known as "coasting" (266). Studies of the efficacy of this approach have been inconclusive (267). Major individual differences in body weight may determine response (268), as may the cotreatment employed to prevent premature luteinization. Because endogenous gonadotropins are suppressed by GnRH antagonists for a limited period of time, less exogenous FSH is required. The ideal day of initiation of gonadotropin therapy is another variable that has been poorly characterized so far (243). The approach of starting exogenous FSH early during the luteal phase of the preceding cycle recognizes the physiological principle of early recruitment of a cohort of follicles for the next cycle (4). However, this protocol did not result in improved ovarian response in women with a low oocyte yield during previous IVF attempts (269).

To allow for the clinical introduction of recFSH, large scale, multicenter, comparative trials in IVF sponsored by pharmaceutical companies were published from 1995 onward (214). Several independent comparative trials have since been published, but sample sizes of these single-center studies were usually insufficient to allow for the detection of small differences. Meta-analyses (270) and health economics studies (271, 272) indicated a slightly improved outcome for recFSH compared with uFSH. A meta-analysis comparing recFSH *vs.* hMG suggested comparable outcomes (273). Subsequent multicenter trials also reported similar clinical outcomes comparing uFSH *vs.* recFSH (274) or hMG *vs.* recFSH (275). A meta-analysis comparing urinary-derived FSH with recFSH showed no significant difference in pregnancy rates (276). Finally, a meta-analysis comparing clinical pregnancy rates per started cycle after recFSH, uFSH, and hMG concluded that there is no evidence of clinical superiority for recFSH over different urinary-derived FSH gonadotropins (277). The data from the principle meta-analyses are summarized in Fig. 4. The continuing debate relating to the relative efficacy and effectiveness of different gonadotropin preparations in IVF is largely driven by commercial rather than scientific imperatives. When selecting a gonadotropin regimen, other factors should therefore be taken into account when selecting a gonadotropin regimen. In terms of tolerance, recFSH preparations showed some improvement over urinary-derived preparations, allowing for safe sc administration (278). The use of recFSH also reduces the theoretical risk of transmission of prion proteins, which have been identified in human urine (279). Although infections by urine prions in humans and animals have not been reported, the risk of prion disease such as new variant Creutzfeldt-Jakob disease has been deemed by some to be sufficient to advise against the use of uFSH, or urinary hCG (uhCG) (280). However, in a recent study of the 143 cases of Creutzfeld-Jakob to date registered in the United Kingdom, 63 were females and only one of these had undergone an infertility treatment from 1998–1999 (281). Although this may suggest low risk in association with infertility treatments, the long incubation period of this condition may continue to mask the real risk.

More recently, a chimeric FSH agonist (so called recFSH-CTP) (220), generated by the fusion of the CTP of hCG (responsible for the prolonged metabolic clearance compared with LH) with the FSH-β chain has been studied in IVF patients. Early studies of this compound showed repeated injections to be safe, with no antibody formation (282). Subsequently, it was demonstrated that a single injection of 120 μg FSH-CTP induced multiple follicle growth similar to that induced by 150 IU recFSH given daily for 7 d (283). The half-life was 60–75 h. When a single dose of long-acting FSH-CTP is given at a dose above the threshold requirements for developing follicles, multiple follicular development occurs. When FSH-CTP levels decline below the threshold, FSH-sensitive follicles cease development and become atretic. This can be prevented by timely institution of daily recFSH injections. The initial report of the birth of a healthy baby, after the single injection of 180 μg in the early follicular phase of the cycle followed by three injections of 150 IU rFSH, demonstrated the feasibility of this approach (284). Moreover, a recent dose-finding study showed that a single dose of FSH-CTP can indeed induce and maintain multifollicular growth for an entire week (285). The total dose of FSH-CTP required to meet criteria for hCG administration (at least

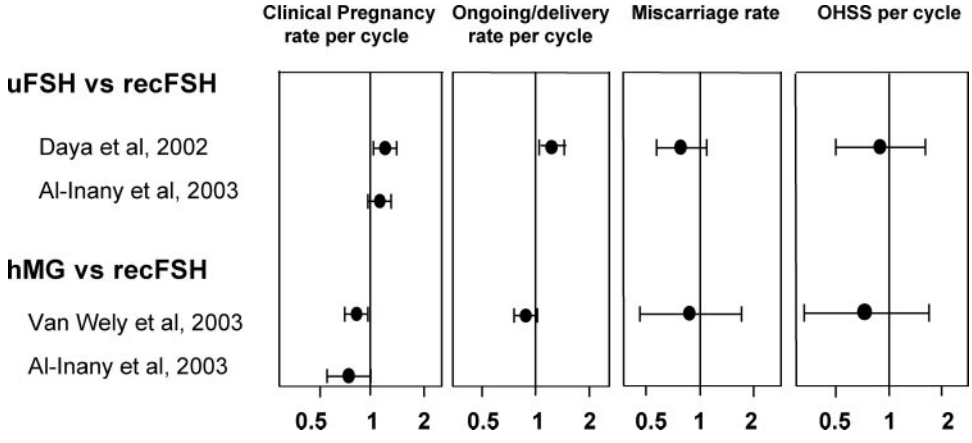

FIG. 4. Overview of results of published meta-analyses comparing uFSH with recFSH and hMG with recFSH for ovarian stimulation in IVF. The *bars* indicate the odds ratios and 95% confidence intervals for the given endpoints. Odds ratios greater than 1 indicate superiority of recFSH.

three follicles with a diameter of at least 17 mm) was similar for doses of 120, 180, and 240 μg, suggesting that the lowest effective dose may be even less than those tested. Studies are now directed at establishing the optimal FSH-CTP dose and regimen for different subsets of IVF patients.

C. The role of LH

A number of recent studies indicated that excessively suppressed LH concentrations in the late follicular phase may be detrimental for clinical IVF outcome (286, 287). Under these circumstances, the use of urinary preparations containing both LH and FSH activity or the addition of recLH or rechCG next to exogenous FSH may be useful. However, several recent studies failed to confirm these findings and questioned the need for exogenous LH (288–295). It remains unclear for which patients this approach may be beneficial. Supplementation of LH activity may offer advantages in some patients by hastening large follicle development and therefore shortening the duration of treatment (296). Moreover, the work of Zeleznik and co-workers (33) referred to a potential therapeutic role for LH in effecting monofollicular stimulation as part of a sequential ovarian stimulation protocol after initiation with recFSH. This concept was supported in a recent study in which anovulatory women with a hyperresponse to recFSH were randomized to continue treatment with the addition of either placebo or recLH (297). In those in whom LH was administered, a trend toward fewer preovulatory follicles was observed. However, in a parallel study, treatment with recLH alone in the late follicular phase was found to be detrimental to preovulatory follicle development (298, 299).

Recently, the concept that exogenous LH is capable of selectively stimulating the development of the more mature dominant follicles has been developed (300). A shift from FSH to LH preparations during stimulation may therefore be useful to stimulate a more homogeneous cohort of follicles for IVF (33, 34). However, opposing views have also been published suggesting no added value of LH supplementation (301). In accordance with the reported association between low LH levels ($<$0.5 IU/liter) and lower ongoing pregnancy rates in IVF cycles (286), LH levels were proposed to have a role in the lower pregnancy rates in GnRH antagonist cycles, because these cycles often lead to extensive suppression of endogenous LH activity during the late follicular phase if combined with rFSH administration. However, several recent studies reported conflicting results with regard to a possible association between serum LH levels during ovarian stimulation and IVF outcomes (302–305). Recently, it was proposed that it might be more appropriate to look at a LH "window" instead of a single LH cutoff level, because there seems to be a "threshold" LH level, below which E_2 production is not adequate, and a "ceiling" level, above which LH may be detrimental to follicular development (290).

The debate as to the optimal LH exposure for successful IVF outcomes continues. A novel approach recently proposed aims to improve outcomes by reducing the incidence of premature LH rises, as has been observed to occur in a small proportion of patients (306). It is suggested that earlier administration of GnRH antagonist could eliminate this problem. In a recent randomized study, neither follicular development nor the number of mature oocytes obtained was adversely affected by commencing GnRH antagonist on d 1 *vs.* d 6 of stimulation. However, LH and E_2 exposure in the follicular phase was reduced in d 1 administration compared with initiation on d 6 of stimulation (307). Previous studies have suggested that prevention of high LH levels at the commencement of stimulation may improve endometrial receptivity (303, 307). Additional studies are required to ascertain the effects of the different GnRH antagonist protocols on endometrial maturation and implantation.

D. GnRH agonists

As outlined earlier, the introduction of GnRH agonists to prevent a premature rise in LH, premature oocyte maturation, and luteinization had a considerable impact on outcomes in IVF. They have now been in use for some 20 yr, yet surprisingly few dose finding studies have been performed (308), and randomized studies comparing different GnRH agonists are scarce. However, much attention has been given to discerning the optimal protocol for their use.

In the long protocol, GnRH agonist treatment usually commences in the luteal phase in the preceding cycle and is continued until hCG administration. Due to the intrinsic agonist activity of the compound, pituitary down-regulation is preceded by an initial stimulatory phase (referred to as the "flare" effect). This flare effect renders the approach of GnRH agonist long protocol for ovarian stimulation time consuming, because ovarian stimulation can only commence when pituitary quiescence has occurred, usually around 2 wk after commencing treatment (309). It is uncertain whether ovarian response to exogenous stimulation is affected by GnRH agonist cotreatment (310), and some women suffer from serious hypoestrogenic side effects, such as mood changes, sweating, and flushes. The "short" or "flare-up" protocol combines GnRH agonist therapy, started at cycle d 2, with gonadotropins initiated 1 d later (311). The immediate stimulatory action of the GnRH agonist serves as the initial stimulus for follicular recruitment. Adequate follicular maturation is on average reached in 12 d, which should allow enough time for sufficient pituitary desensitization to prevent any premature LH surges (312).

Several investigators have tried to shorten the duration of GnRH agonist administration by early cessation, because pituitary recovery after cessation takes around 14 d (313). The GnRH agonist is started in the midluteal phase of the preceding cycle and discontinued during or even before the FSH treatment is started. Several prospective randomized controlled studies have been performed comparing this approach with the long protocol (314–317). Although premature rises in LH did not occur (confirming delayed pituitary recovery from desensitization), no clear clinical benefit has been demonstrated by this approach.

A meta-analysis comparing short and long IVF protocols showed a higher number of oocytes retrieved and higher pregnancy rates in the long protocol, although more units of gonadotropin were needed (318). In terms of gonadotropin suppression and number of retrieved oocytes, the midluteal phase of the preceding cycle is the optimal moment for the

initiation of the GnRH agonist, in comparison to the follicular, early, or late luteal phase (319, 320). A major clinical advantage of the long protocol of GnRH agonist administration is the contribution to the planning of the oocyte retrieval because the initiation of exogenous gonadotropins after pituitary desensitization can be delayed, without a detrimental effect on IVF outcome (321, 322). A potential disadvantage with the luteal phase initiation of GnRH agonist is that spontaneous pregnancy present at the time of commencing treatment cannot be excluded with certainty. The extensive evidence supporting the long protocol has led to its widespread adoption as the standard of care (318). However, the recent clinical introduction of GnRH antagonists may ultimately lead to a new standard of care in IVF practice.

E. GnRH antagonists

GnRH antagonists may be administered at any time during the early or midfollicular phase of a treatment cycle to prevent a premature LH rise. Several studies have been performed to determine the minimal effective dose and treatment schedule in IVF patients (323–325). Two general approaches have emerged. In the single-dose protocol, one injection of 3 mg cetrorelix (ganirelix is not provided in this depot formulation) is administered in the late follicular phase on stimulation d 8 or 9. This is sufficient to prevent a LH surge in 80% of women (324). In the multiple-dose GnRH antagonist protocol, 0.25 mg cetrorelix or ganirelix is given daily from the sixth day of gonadotropin stimulation onward (323, 325). The rationale behind starting GnRH antagonist at least 5 d after commencing stimulation with gonadotropins is based on the reduced possibility of observing a premature LH rise in the early follicular phase (326).

Four large, industry-sponsored, prospective multicenter clinical trials comparing daily GnRH antagonist injections with long GnRH agonist protocols in IVF patients undergoing ovarian stimulation have been reported (327–330). With a GnRH antagonist, the duration of gonadotropin treatment is shortened by 1–2 d, and slightly fewer follicles are seen at the time of hCG injection compared with a GnRH agonist. Therefore, the number of recovered oocytes tends to be lower. In these studies, no significant difference was found with respect to percentages of metaphase II oocytes, fertilization rates, and number of good quality embryos. Pregnancy rates were adequate in both groups in all four studies, but in every one the absolute rate was lower in the GnRH antagonist group. A meta-analysis of five large randomized trials showed an overall decrement in pregnancy rate of 5% (odds ratio, 0.75; 95% confidence interval, 0.62–0.97) (331) (Table 1). It has been hypothesized that the lower observed pregnancy rates may be a consequence of the currently advised treatment regimen. It has been suggested that the larger numbers of oocytes and embryos with agonists allow better selection, although the numbers of good quality embryos do not seem to be different. The GnRH antagonist was started on a fixed day of stimulation (d 6) in these studies, which may be too early for some patients and may lead to a diminished number (and quality) of oocytes.

Studies comparing the fixed antagonist protocol with a flexible protocol, in which the daily antagonist administration is started when at least one follicle reached a size of 14 mm, showed no differences in IVF outcome, except that the dose of GnRH antagonist was reduced in the flexible protocol (332). When GnRH antagonist is commenced, there appears to be no requirement to increase the dose of FSH (264, 333, 334) or supplement LH (335). Commencing GnRH antagonist in the late follicular phase enables the endogenous FSH rise to be harnessed to commence ovarian stimulation and then supplemented by exogenous gonadotropin stimulation from the midfollicular phase onward to achieve multifollicular development (336). The concept of thus "extending the FSH window" is illustrated in Fig. 1. This novel approach promises a cost-effective and patient-friendly alternative to standard stimulation regimens.

Based on the inverse association between implantation rates and ganirelix dose in the higher dosage groups in the large dose-finding study (337), direct effects of GnRH antagonists on human embryos have been suggested. Adverse effects were not observed on the freeze-thaw embryos of these cycles (338). Moreover, retrospective comparison of pregnancy rates after transfer of frozen-thawed two-pronucleate oocytes obtained in either a long GnRH agonist protocol (n = 286) or a GnRH antagonist protocol (n = 56) showed no differences in implantation, pregnancy, or miscarriage rates (339).

V. Adjuvant Therapies

The aim of adjuvant therapies is to improve the efficacy of ovarian stimulation by reducing the dose of gonadotropin required to effect the same response or improve response to gonadotropin stimulation, or by simplifying treatment protocols.

A. Oral contraceptive pretreatment

The use of exogenous sex steroids to manipulate secondary follicle recruitment has been proposed since the early

TABLE 1. Principal results of meta-analysis of randomized studies comparing IVF outcomes after cotreatment with GnRH antagonist or GnRH agonist during ovarian stimulation (331)

Outcome parameter	Odds ratio	95% Confidence intervals
Duration of ovarian stimulation (d)	−1.12[a]	−1.45 to −0.80
Premature LH surge	1.76	0.75 to 4.16
No. of oocytes retrieved per cycle	−1.86[a]	−2.47 to −1.25
Clinical pregnancy rate per oocyte retrieval	0.79	0.63 to 0.99
Clinical pregnancy rate per embryo transfer	0.76	0.60 to 0.97
Miscarriage rate	1.03	0.52 to 2.04
Incidence of severe OHSS	0.47	0.18 to 1.25

[a] Weighted mean difference.

days of IVF. Early studies had demonstrated that oral administration of ethinyl E_2 in the early follicular phase of the menstrual cycle led to suppression in gonadotropins and a lengthening of the follicular phase (340, 341). Subsequent studies in rhesus monkeys confirmed this observation and demonstrated the presence of a highly sensitive feedback relationship between E_2 and FSH during the follicular phase of the menstrual cycle (342). This concept found ready application in dealing with one of the principal problems associated with ovarian stimulation for IVF: the premature LH surge and luteinization. The perceived need to allow programming of oocyte retrieval led to a number of studies addressing the role of oral contraceptives (OCs). Fixed schedule protocols were developed by a number of groups in which OCs were administered in advance of ovarian stimulation and planned follicle aspiration. The preparations studied varied from combined estrogen-P pills (343) to P-only treatment (344). The optimal duration of treatment was debated, with some studies suggesting that prolonged treatment with OCs was associated with excessive suppression, prolonged ovarian stimulation, and increased cancellation rates (345, 346), whereas others could demonstrate no such detrimental effects of prolonged pretreatment (343). These and other studies of OCs as a tool for manipulating the length of the follicular phase promised to turn oocyte retrieval from an emergency to an elective operation (347). Despite their apparent efficacy, ease of administration, and fewer side effects, subsequent randomized studies comparing OCs to GnRH agonists as a means of preventing premature luteinization showed the superiority of the latter, and because of this, OCs are no longer widely used for this indication.

To facilitate the planning of the initiation of exogenous gonadotropins in a GnRH antagonist cycle, independent of the menstrual period, OC pretreatment has been evaluated in a number of small studies (348). Although there is evidence that OC pretreatment may aid in the scheduling of IVF cycles when GnRH antagonists are employed, more prospective studies are required to evaluate the effect on IVF outcome.

It has been proposed that antral follicles at a more advanced stage of development may be so responsive to FSH that they may start their development during the late luteal phase. This may lead to a number of follicles reaching maturity and postmaturity ahead of the rest of the cohort. The concept of synchronizing the cohort of antral follicles before ovarian stimulation by administering exogenous steroids has been put forward as a means of ensuring that the entire cohort of follicles reaches maturity at the same moment, and therefore improving IVF outcomes. Indeed, a number of studies have demonstrated that induction of a hypogonadotropic state with synthetic steroids may synchronize follicle development (344, 349). Recently, it was demonstrated that luteal administration of E_2 can also reduce size discrepancies of antral follicles (350). The authors proposed that synchronization of follicular growth allows ovulation to be triggered when the majority of follicles have reached concomitant maturation. This concept remains untested in the context of randomized clinical trials.

The potential role for OCs in improving outcomes in high responders has also been addressed. Women with polycystic ovary syndrome (PCOS) are at increased risk of developing OHSS. The frequently observed excessive ovarian response of these patients to ovarian stimulation presents a therapeutic challenge. Predicated on the concept that women with PCOS may take longer to achieve down-regulation with GnRH agonist (351), combined down-regulation protocols of GnRH agonist and OCs have been proposed (352). The efficacy of this approach awaits testing by randomized control trials.

B. Insulin-sensitizing agents

The contention that insulin resistance may play a key role in the pathogenesis of ovarian dysfunction in PCOS patients led to the application of insulin-sensitizing agents to induce ovulation and improve outcomes from ovarian stimulation for IVF. The most extensively studied insulin-sensitizing drug in the treatment of anovulation is metformin. Metformin (dimethylbiguanide) is an orally administered drug used to lower blood glucose concentrations in patients with noninsulin-dependent diabetes mellitus (353). It is antihyperglycemic in action, and increases sensitivity to insulin by inhibiting hepatic glucose production and by increasing glucose uptake and utilization in muscle. These actions result in reduced insulin resistance, lower insulin secretion, and reduced serum insulin levels. Metformin has been used for many years for the treatment of diabetic patients and appears to be safe for long-term use. It has not been associated with an increased risk of congenital abnormalities in diabetic women who subsequently became pregnant (354). *In vitro* studies have not demonstrated teratogenicity.

The main side effects of metformin are nausea and diarrhea, which may occur in 10–25% of patients and contribute to the weight loss effects observed with metformin. If these symptoms persist despite lowering the dose, alternative therapy should be given. For this reason, metformin should be given in a low-start rising-dose regimen. Rarely, lactic acidosis may occur (0.3 episodes per 10,000 patient years) (355) if hepatic or renal disease is present, and these should be excluded before commencing therapy.

Troglitazone is a thiazolidinedione, one of a group of insulin-lowering oral drugs that have also been studied in the context of inducing ovulation in women with PCOS. A large randomized study suggested that troglitazone may be an effective ovulation induction agent (356). However, numerous reports of fatal liver toxicity have led to its withdrawal by the U.S. Food and Drug Administration. Its safety with respect to potential teratogenicity and long-term effects on children have not yet been demonstrated.

Three studies have evaluated the effect of metformin treatment before IVF. In a retrospective study of 46 women with PCOS undergoing 60 cycles of IVF treatment, metformin-treated women had more mature oocytes, increased fertilization rates, and higher clinical pregnancy rates than controls (357). In a small, open-label, randomized crossover trial, metformin increased the number of oocytes collected among insulin-resistant, obese women with PCOS (358). However, in a recent randomized double-blind study in which women with polycystic ovaries were treated for at least 16 wk, no differences were found in duration of FSH stimulation, num-

ber of oocytes, fertilization rates, embryo quality, or pregnancy rates (359). Because benefits in subgroups of women with PCOS have not yet been demonstrated, caution should be applied before metformin is prescribed in the context of adjuvant treatment for IVF.

C. Aromatase inhibitors

Aromatase inhibitors have been in clinical use for more than 20 yr, primarily in the treatment of postmenopausal patients with advanced breast cancer (360). The more recently developed third generation of aromatase inhibitors are characterized by their potency in inhibiting the aromatase enzyme without significantly inhibiting other steroidogenesis enzymes. Moreover, they are active when taken orally and show rapid clearance from the body, with a half-life of around 45 h (361). One of the third-generation compounds, letrozole, has been the focus of study as a potential therapeutic agent for induction of ovulation (362). Rather than antagonizing estrogen feedback activity at the hypothalamic-pituitary axis as with CC, this approach reduces the amount of estrogens being synthesized. Aromatase inhibitors block the conversion of androstenedione and testosterone to estriol and E_2, respectively (363), reducing estrogenic feedback at the pituitary/hypothalamic axis, increasing gonadotropin secretion, and thereby stimulating growth of ovarian follicles (362).

Early follicular phase administration of letrozole was shown in monkeys to stimulate follicle development (364). Subsequent small clinical studies employing a dose of 2.5 mg/d from d 3 to d 7 of the menstrual cycle have suggested that it may be an effective ovulatory agent in CC-resistant women (362). A local effect at the ovary to increase sensitivity to FSH by blocking the conversions of androgens to estrogens has also been proposed, because accumulating intraovarian androgens may increase FSH receptor gene expression (365). On the other hand, significantly increased intraovarian androgen/estrogen ratios may also induce follicle atresia.

Studies have shown that when given at doses of 5 mg/d, letrozole causes a marked reduction in E_2, estrone, and estrone sulfate, with minimal, primarily gastrointestinal, side effects (366, 367). Although theoretically attractive as a means of reducing the dose of exogenous gonadotropins required for ovarian stimulation in IVF, no randomized studies have been published as yet. A recent nonrandomized study involving ovarian stimulation with intrauterine insemination appeared to indicate that the dose of FSH required was less when letrozole was given from d 3 to 7 of the cycle before commencing FSH administration (368).

A further possible role for aromatase inhibitors that remains under investigation is to reduce E_2 levels during ovarian hyperstimulation with gonadotropins. The hypothesis being tested is that by preventing excessive E_2 synthesis, less disruption of endometrial receptivity is likely. As with the other possible roles for aromatase inhibitors, future studies must determine whether these approaches are of value and are safe for ensuing pregnancy.

D. Growth Hormone

In addition to the earlier described rodent studies, there is evidence that GH enhances ovarian steroidogenesis and follicular development by increasing the sensitivity of the ovaries to gonadotropin stimulation (369, 370). GH has therefore been proposed as an adjuvant therapy to ovarian stimulation for IVF. Studies addressing this possibility remain scarce, however. When GHRH was administered to women undergoing IVF, no improvement was observed in the ovarian response to FSH, although a significant rise in GH and IGF-I was observed (371). However, in a further study, the addition of GH to gonadotropin therapy in hypogonadotropic patients reduced the gonadotropin dose required to achieve ovulation (372). In a recent meta-analysis, a small but significant improvement in pregnancy rates appeared to be associated with GH supplementation (373). However, caution is required in interpretation due to the small number and quality of studies available.

Studies of patients with GH deficiency have suggested that the therapeutic value of GH may be limited. An example of such an "experiment of nature" is the infertile patient suffering from a GH deficiency (Oliver McFarlane syndrome). In one reported case, adjuvant GH did not influence the ovarian response to exogenous gonadotropins (374). In Laron-type dwarfism (where low IGF-I and normal GH levels are observed, due to deficiency of GH receptors), spontaneous and ART pregnancies have been reported (375, 376). Recently, ovulation induction and successful pregnancy after gonadotropin therapy were reported in two women with combined pituitary hormone deficiency secondary to *Prop 1* gene mutations (377). In these women, neither GH, IGF-I, or prolactin appeared necessary for ovulation, embryonic development, or normal pregnancy outcome. These data serve to illustrate the redundancy evident in the endocrine and paracrine control of follicular development and function.

E. Androgens

Androgens act as paracrine regulators of follicular maturation and atresia modulating gonadotropin action on granulosa cells through amplification of cAMP-mediated postreceptor signaling (378). Although their action in promoting FSH-induced granulosa cell differentiation points to a potential therapeutic role (378), few studies have addressed androgens as adjuvants to ovarian stimulation for IVF.

It has long been believed that hyperandrogenic states such as PCOS are associated with poorer outcomes from IVF. In a recent meta-analysis, PCOS patients were found to produce more oocytes in response to stimulation, but these showed more reduced fertilization rates than oocytes derived from non-PCOS patients. Pregnancy rates did not appear to be affected (379). No randomized studies of androgen supplementation have been carried out, but data from retrospective studies suggest that low levels of testosterone (<20 ng/dl) are predictive of poor cycle outcome (380). More data from well-designed studies are required to determine the potential role of androgen supplementation in IVF stimulation protocols.

VI. Sequelae of Ovarian Stimulation

A. Effects on corpus luteum function

From the first attempts at IVF in the early 1970s, it was clear that ovarian stimulation with hMG disrupted the luteal phase (198). Initial studies in the United States in 1983 concerning hMG-stimulated IVF cycles also confirmed the occurrence of an abnormal luteal phase in IVF cycles with characteristic features of elevated P levels during the early luteal phase along with a significantly reduced luteal phase length (381) (Fig. 5).

With the adoption of GnRH agonist cotreatment for the prevention of a premature rise in LH, it became apparent that recovery of the pituitary from down-regulation during the luteal phase was slow (resulting in a lack of support of the corpus luteum by endogenous LH (241, 313, 314). It was observed shortly thereafter that the corpus luteum could be rescued by the administration of hCG (382–384), and this treatment modality became the standard of care for luteal support during the late 1980s. A meta-analysis combining results from 18 randomized trials showed increased IVF pregnancy rates with hCG supplementation (385). However, 5% of hCG-supplemented patients developed OHSS. Because of this association between hCG and OHSS (386), luteal phase hCG support has been largely replaced over the years by luteal phase P supplementation (387).

Attempts to secure pituitary recovery during the luteal phase by the early follicular phase cessation of GnRH agonist cotreatment (314, 317, 388) failed, presumably due to the fact that other mechanisms are also involved in suppression of pituitary function during the luteal phase. Because of the rapid recovery of pituitary gonadotropin release after discontinuation of GnRH antagonist (31, 389), it has been speculated that luteal phase supplementation may not be required after the late follicular phase administration of GnRH antagonist (390). However, various studies in IVF applying GnRH antagonist cotreatment have now clearly shown that luteolysis is also initiated prematurely, resulting in a significant reduction in the length of the luteal phase along with greatly compromised chances for pregnancy (391–394). Other mechanisms may explain the nonphysiological endocrine milieu observed when this regimen is employed (Table 2). The hCG administered for inducing the final stages of oocyte maturation has a much longer half-life than native LH (395). Multiple corpora lutea resulting from multiple dominant follicle development during ovarian stimulation are supported by the profound luteotropic activity of the midcycle hCG bolus. Supraphysiological serum P or E_2 concentrations in the early luteal phase may elicit a more profound suppression of pituitary LH and FSH secretion than occurs in the natural cycle (2, 396, 397).

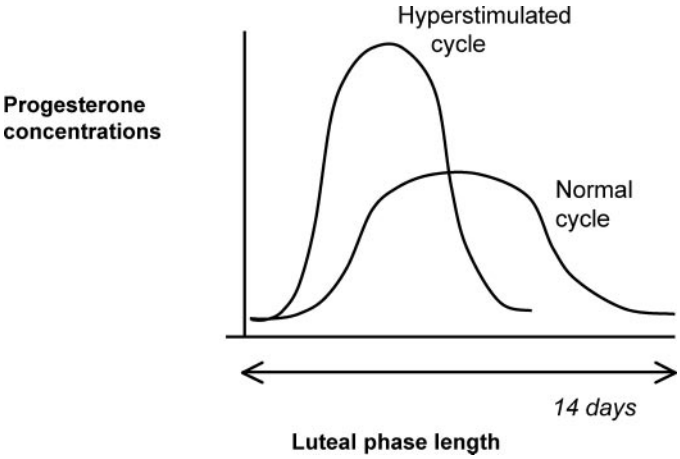

FIG. 5. Schematic representation of changes in luteal phase length and endocrine profile induced by ovarian hyperstimulation for IVF. [Published with permission from H. W. Jones: *Hum Reprod* 11(Suppl 1):7–24, 1996 (381). © The European Society of Human Reproduction and Embryology. Reproduced by permission of Oxford University Press/Human Reproduction.]

Recently, more detailed studies have confirmed that early- and midluteal phase LH levels remained suppressed after the follicular phase administration of GnRH antagonist (394, 398). Luteolysis has been found to be advanced in the non-supplemented luteal phase, whether final oocyte maturation is induced with recLH, rechCG, or LHRH agonist in GnRH antagonist cycles (394). These findings are consistent with studies of ovarian stimulation in monkeys and in humans showing that P levels decline in the luteal phase in association with the fall in circulating hCG (317, 399). There is increasing evidence that the short luteal phase after ovarian stimulation is therefore due to the decay in hCG levels administered at midcycle and the continued potent feedback suppression of pituitary LH secretion by the supraphysiological serum levels of E_2 and P. The clinical consequence is that all cycles undergoing superovulation require luteal supplementation (2, 400).

1. Luteal support. Luteal phase length can be restored by 1) stimulating the corpora lutea with hCG (luteal phase support), or 2) supplementing the luteal phase with steroids, such as estrogen and P (luteal phase supplementation). Recent preliminary observations suggest that corpus luteum function can also be maintained by small repeated doses of GnRH agonist (401).

hCG can be administered during the luteal phase in doses of 1,500/2,500 IU at d 3, 6, and 9 after inducing ovulation, or 1,500 U on alternate days (402). In the case of luteal phase supplementation, P is administered at different dosages such as 25 and 50 mg daily im. Micronized P can also be administered intravaginally at a dose ranging from 300 to 600 mg daily or as a vaginal gel at a dose of 90 mg daily (403). Oral estrogen can be added in case P is used (404).

Approximately 30 randomized controlled trials have been published to compare the different drugs used for luteal

TABLE 2. Possible mechanisms underlying the abnormal luteal phase after ovarian stimulation for IVF

Mechanisms
Slow recovery from pituitary down-regulation by GnRH agonists
Exaggerated ovarian feedback in response to stimulation on hypothalamic-pituitary function
Direct effect at pituitary of hCG bolus given to trigger final oocyte maturation
Removal of large quantities of granulosa cells during retrieval of cumulus-oocyte complexes
Negative feedback by high early luteal phase sex steroid levels at pituitary

phase supplementation and support (404). Intramuscular P appeared to be marginally superior to oral or vaginal use. A decrease in pregnancy rates after the use of oral P compared with hCG administration has been reported (405, 406). Support with hCG or supplementation with P did not reveal a difference in pregnancy rates (407). The addition of E_2 valerate to P supplementation has been analyzed, but studies so far remain inconclusive as to the value of this (408). Insufficient data are currently available to analyze whether the addition of hCG to P administration has any benefit (409).

Because of the potential risk of enhancing the occurrence of OHSS, the administration of hCG is not advisable. In summary, the available data in this field point to P supplementation as the preferred means of providing luteal support. Although im P may be more effective than the intravaginal route, the pain and inconvenience of daily deep injections renders the vaginal approach preferable in practice. The optimal timing of initiation and duration of treatment remains to be fully clarified. However, a randomized controlled study comparing administration of vaginal P for 14 d after oocyte pickup, with extended administration for 3 wk after a positive pregnancy test, revealed no difference in outcomes (410).

Studies of luteal function in early pregnancy have shown that serum P levels are markedly raised for up to 9 wk gestation compared with spontaneous pregnancy (411). 17-Hydroxyprogesterone, the principal P produced by the corpus luteum, remains the dominant P after IVF throughout the first trimester. This is in contrast to spontaneous pregnancies, where P of trophoblast origin is secreted in higher concentrations than 17-hydroxyprogesterone from 5 wk gestation (411). It is clear that the multiple corpora lutea produced during IVF produce high levels of P in early pregnancy, and additional supplementation during this phase is probably superfluous.

B. Effects on endometrial receptivity

The nonphysiological hormonal milieu associated with ovarian stimulation is widely held to be detrimental to endometrial receptivity (Table 3). The fine balance of endocrine and paracrine factors involved in the preparation of the endometrium to allow implantation is disrupted, primarily as a result of excess estrogen levels. Early studies of the effect of ovarian stimulation reported an association between delayed postovulatory endometrial maturation and defective induction of PRs (412). Although E_2 receptor expression appeared unchanged, stimulation with CC and hMG was shown to significantly reduce the number of cytosolic PRs when compared with a control group (413). Moreover, a premature reduction in PRs in the early luteal phase has been found after ovarian stimulation (414). A further study, in which endometrial biopsies were performed close to the implantation period, revealed a significant reduction in the nuclear receptor level in both the glands and the stroma for P and E_2 receptors (400, 415).

TABLE 3. Possible mechanisms underlying abnormal endometrial receptivity after ovarian stimulation for IVF

Mechanisms
Disrupted early follicular phase endocrinology
Suboptimal timing of hCG bolus to trigger final oocyte maturation
Abnormal luteal phase steroid levels
Inadequate luteal support
Direct effect of GnRH analogs

The principal mechanism by which ovarian stimulation is considered to reduce endometrial receptivity is through exposure of the endometrium to supraphysiological levels of E_2 (416). Elevated estrogen concentrations may increase sensitivity to P action and thus lead to secretory advancement (417). Studies in humans (418) and rodents (419) have indicated that the magnitude of the E_2 dose to which the endometrium is exposed in the late follicular and early luteal phase affects the duration of the receptive phase.

Studies in which endometrial biopsies were taken during GnRH agonist/gonadotropin stimulation in the preovulatory phase have shed further light on the impact of ovarian stimulation on endometrial histology at the end of the follicular phase. Histological findings before hCG injection demonstrated accentuated proliferative aspects and early secretory changes, which occurred before any P rise was observed (420). When endometrial biopsies were taken on the day of oocyte retrieval in IVF cycles, endometrial advancement was observed in more than 90% of patients (421). If the advancement exceeded 3 d, no pregnancy was observed (400). Biopsies taken 7 d after ovulation show endometrial delay or glandular-stromal dissociation. Apparently, stimulation with GnRH agonist and gonadotropins induces early endometrial advancement with subsequent glandular maturation arrest in the midluteal phase (422). Similar studies have been carried out in women stimulated with a combination of GnRH antagonist and recFSH. Endometrial biopsies performed at the day of oocyte pickup again showed advancement on histological analysis, and no pregnancy was established if histology on light microscopy was more than 3 d out of phase (418).

The endocrinology of the early follicular phase has also been shown to affect the luteal phase. In one study, high exposure of the genital tract to LH and E_2 in the early follicular phase was shown to be associated with a reduced chance of pregnancy (423). These findings were consistent with the concept that it is the duration of P exposure rather than the actual concentrations of E_2 and P that is crucial for endometrial receptivity, provided that a threshold level of E_2 is exceeded and PRs in endometrium are induced (424). If increased E_2 levels are present in the early follicular phase, this threshold is reached earlier, extending the period of P action before hCG administration (423).

Late follicular endocrine manipulation has also been shown to impact on endometrial receptivity. In a study comparing outcomes when hCG was administered when follicle size met standard criteria or was delayed by a further 2 d, a higher incidence of endometrial advancement [defined by Noyes' criteria (111)] on the day of oocyte retrieval was observed (425).

The administration of CC in normo-ovulatory women is associated with reduced size and number of glands in the endometria (194). To counteract the antiestrogenic effect of CC, the use of ethinyl-E_2 in this respect has been studied in

a randomized controlled trial, and significantly improved pregnancy rates were reported (426).

Periimplantation events remain poorly understood. If effective interventions to aid implantation are to be designed, a further search for unrecognized factors involved in implantation in stimulated cycles is required. Global gene expression offers an additional novel and powerful means of analyzing the impact of ovarian stimulation on the endometrium. Initial studies have shown multiple differential gene expression when comparing the follicular to the secretory phases (126–129) and when comparing the luteal phase of the spontaneous cycle to that after ovarian stimulation (427, 428) (Table 4).

C. Effects on embryo quality

The use of exogenous gonadotropins for ovarian stimulation has been reported to affect embryo development at a number of different stages. This is particularly well characterized in the mouse, where ovarian stimulation is reported to both a decrease (429) and delay (430) in development of one or two-cell embryos to blastocysts *in vitro*. Ovarian stimulation has also been reported to delay the *in vivo* development of embryos (430, 431). It has also been reported that ovarian stimulation reduces the number of cells and of microvilli on the blastocyst (432). Ovarian stimulation also results in a delay in implantation and a decrease in the expression of the angiogenic factor VEGF at implantation sites (429, 430) and has been associated with increased postimplantation mortality in mice (429–431, 433). Furthermore, ovarian stimulation was associated with a reduction in fetal growth and a prolonged gestation period (434). Studies in mice revealed no significant impact of ovarian stimulation on blastocyst expression of the genes for IGF-II, IGF-II receptor, or VEGF. However, a number of studies reported that exogenous gonadotropin treatment increases the frequency of chromosomal abnormalities (435, 436), and this may underlie the impaired *in vitro* embryo development after ovarian stimulation.

Little data are available in the human embryos on the direct effects of ovarian stimulation. A small *in vitro* study of human embryo adhesion rates suggested that high E_2 levels were deleterious primarily due to a toxic effect on the cleavage stage embryo (437). However, no negative impact on embryo quality was reported in a study of oocyte and embryo quality in women with excessive response to ovarian stimulation (438). In a recent retrospective study comparing embryo quality in the natural *vs.* stimulated IVF cycle, no differences in the cleavage capacity or quality assessment of the embryos were observed (439). Exposure to high FSH levels may also have direct consequences for embryo development. *In vitro* studies in which oocytes from preantral follicles were cultured in FSH-supplemented medium showed no beneficial effect (440). In combination with insulin, the presence of FSH appeared to be detrimental to oocyte development and to promote inappropriate granulosa cell differentiation (441). These findings may have implications for the current paradigm of maximal ovarian stimulation for IVF to obtain large numbers of oocytes at pickup. New developments in genomic and proteomic analyses, together with increasing knowledge derived from intervention

TABLE 4. Genes up- and down-regulated by more than a factor of 10 in the stimulated (day of hCG + 7) *vs.* nonstimulated (day of LH peak + 7) cycle

Up-regulated genes			Down-regulated genes		
Name	Fold-change	Category	Name	Fold-change	Category
Troponin C	30.89	Structural protein	Dipeptidylpeptidase 4 (CD26, adenosine deaminase complexing protein 2)	54.37	Immune response
Matrix metalloproteinase 26	16.96	Enzyme	Dipeptidylpeptidase 4 (CD26, adenosine deaminase complexing protein 2)	43.48	Immune response
Sorbitol dehydrogenase	15.79	Enzyme	Thrombomodulin	24.38	Coagulation factor
Calpain 6	13.56	Glycoprotein	Leukemia inhibitory factor (cholinergic differentiation factor)	23.02	Cytokine
Major histocompatibility complex, class II, DO β	12.23	Immune response	Mucin 16	13.61	Membrane protein
Differentially expressed in hematopoietic lineages	11.89	Inhibitor	Dipeptidylpeptidase 4 (CD26, adenosine deaminase complexing protein 2)	13.58	Immune response
Serine (or cysteine) proteinase inhibitor, clade A (α-1 antiproteinase, antitrypsin), member 5	11.88	Inhibitor	Cytochrome P450, family 3, subfamily A, polypeptide 5	12.96	Energy transduction
Galanin	11.79	Neuropeptide	Glutathione peroxidase 3 (plasma)	12.51	Enzyme
Branched chain keto acid dehydrogenase E1, β polypeptide (maple syrup urine disease)	10.32	Enzyme	IGF binding protein 1	11.99	Regulatory protein
			ATP-binding cassette, subfamily C (CFTR/MRP), member 3	11.12	Transporter
			Glutathione peroxidase 3 (plasma)	11.12	Enzyme
			Solute carrier family 15 (oligopeptide transporter), member 1	10.62	Transporter

Data are from Ref. 428.

studies such as those ongoing on the impact of various ovarian stimulation regimens on aneuploidy rates in IVF embryos (442), will lead to further clarification.

D. Side effects and complications

Complications related to invasive IVF procedures, such as oocyte retrieval and embryo transfer, predominantly involve infection and bleeding along with anesthesia problems (443). The drawbacks associated with profound ovarian stimulation for IVF include considerable patient discomfort such as weight gain, headache, mood swings, breast tenderness, abdominal pain, and sometimes diarrhea and nausea (444). In this respect, it is important to appreciate that after a first unsuccessful IVF attempt, around 25% of patients refrain from a second cycle, even in countries where costs are covered by health insurance companies (445).

1. Ovarian hyperstimulation syndrome. OHSS is a potentially life-threatening complication characterized by ovarian enlargement, high serum sex steroids, and extravascular fluid accumulation, primarily in the peritoneal cavity. In severe cases, hypotension, increased coagulability, reduced renal perfusion, and oliguria may occur. Deranged liver function tests, venous and arterial thrombosis, renal failure, and adult respiratory distress syndrome can ensue, and fatalities have been reported (91, 446) (Table 5). The etiology of OHSS is related to the increased LH, FSH, hCG, and E_2 levels associated with ovarian stimulation (447). These cause an increase in expression of VEGF (448), which, being a potent inducer of vascular permeability, can lead to the extravasation of excessive protein-rich fluid. Serum and follicular levels of VEGF are higher in IVF patients who develop OHSS. However, pretreatment serum VEGF levels are not predictive of individual OHSS risk (449). Recent studies of patients with activating mutations of the FSH receptor (450) or LH/hCG receptors (451) that result in spontaneous OHSS have highlighted the role of gonadotropins as initiators of this condition.

TABLE 5. Key features of OHSS

Key features
Etiology
Increased secretion or exudation of protein-rich fluid from ovaries or peritoneal surfaces
Increased follicular fluid levels of prorenin and renin
Angiotensin-mediated changes in capillary permeability
Risk factors
Young age
Low body weight
PCOS
High doses of exogenous gonadotropins
High or rapidly rising serum E_2
History of previous OHSS
Clinical signs
Abdominal discomfort
Abdominal distention
Nausea
Vomiting
Diarrhea
Prevention strategies
Mild stimulation regimens
Puncture of excess follicles
"Coasting"
Cryopreservation of available embryos and transfer in subsequent nonstimulated cycle
Outpatient management
Monitor weight and abdominal circumference
At least 1 liter/d oral fluids
Light mobilization
Regular assessment
Hospital management
Intravenous fluid replacement
Careful fluid balance monitoring
Light mobilization
Thromboprophylaxis

Mild forms of OHSS constitute around 20–35% of IVF cycles, moderate forms 3–6% of cycles, and severe forms 0.1–0.2% (91, 266). Moderate to critical OHSS is very rare after CC treatment but constitutes an important complication of gonadotropin use. The risk is further increased when adjuvant GnRH agonist treatment is employed (452). To some extent, patients at risk of developing OHSS may be recognized by the following features: young age, PCOS, profound hyperstimulation protocols with GnRH agonist long protocol cotreatment, large numbers of preovulatory Graafian follicles, high serum E_2 levels, a high (>5,000 IU) bolus dose of hCG needed to induce final oocyte maturation, the use of hCG for luteal phase supplementation, and finally, the occurrence of pregnancy. The incidence of OHSS is directly related to hCG concentrations, with a 2- to 5-fold increased incidence in case of multiple pregnancy. Preventive strategies in case of imminent OHSS include cessation of exogenous gonadotropins for several days (referred to as "coasting"), cancellation of the IVF cycle, and withholding hCG (91, 266, 453, 454). Additional preventative measures include follicular aspiration, alternative means of inducing oocyte maturation (such as the induction of an endogenous LH surge by the administration of a single bolus dose of GnRH agonist or the administration of the short half-life preparation recLH rather than hCG), prevention of pregnancy during the stimulation cycle by cryopreserving all embryos, or the prophylactic infusion of glucocorticoids or albumen (455).

With regard to GnRH antagonists and OHSS, the results of comparative studies have been inconclusive. Although three studies demonstrated decreased OHSS incidence when a GnRH antagonist was used (327, 330, 456), one study showed a higher incidence of OHSS (329). A meta-analysis that compared the five large comparative studies showed significantly lower OHSS after GnRH antagonists than after GnRH agonists (Table 1).

2. Venous thromboembolism (VTE). The clinical association between VTE and IVF arises primarily within the context of OHSS, in which thromboembolic complications may have fatal consequences. Occasionally patients presenting for IVF treatment may have a previous history of VTE or be considered to be at increased risk of developing thromboembolic complications as a result of undergoing IVF treatment. VTE is a rare complication of ovarian stimulation for IVF. Recent data point to an incidence of 1.6 events per 100,000 cycles/woman (457) and the majority of cases of VTE reported in the literature are associated with the presence of risk factors for thromboembolic disease (458). Ovarian stimulation results in a hyperestrogenic state, which has been associated with hypercoagulability and increased risk of deep vein thrombosis

(DVT) after OC pill use and pregnancy. However, a number of studies have shown changes in coagulation parameters during IVF treatment to be modest (459). During IVF treatment, the action of coagulation factors seems to be associated less with the level of serum E_2 concentrations (which may reach levels 10 times higher than in physiological cycles) than with the biochemical changes that occur after the triggering of final oocyte maturation with hCG. Hyperestrogenism related to ovarian stimulation is not associated with the coagulation abnormalities observed with high estrogen-content OCs, and therefore does not significantly increase the potential for thrombus formation. In contrast, the period after hCG administration reveals clinically significant alterations in the coagulation and fibrinolytic systems.

Clinical reports of DVT occurring after IVF treatment indicate that it most frequently presents in association with OHSS (459) or in early pregnancy between 5 and 10 wk after hCG administration (458). This has implications for both the duration for which prophylaxis should be administered in high-risk patients and the duration for which clinical surveillance should be maintained, to ensure early detection and treatment.

Many reported cases of DVT after IVF are in sites other than the lower limb, but this may simply reflect publication bias. The jugular vein appears to be a relatively frequent site, with the majority of thromboses occurring here being associated with hormonal ovarian stimulation (460)

3. Multiple pregnancy. The frequency and consequences of multiple pregnancies arising from IVF remain a major indictment of the organization and practice of IVF worldwide. Twin birth rates in the United States increased by 75% between 1980 and 2000 and currently represent around 3% of total births (Fig. 6) (461, 462). Similar trends have been reported in European countries (463). Although an association between increased female age and multiple gestation is clearly established, the delay in childbearing accounts for no more than 30% of the observed overall increase in multiple pregnancies (464). Although the available data indicate that the majority of twin births are still unrelated to infertility therapies (3, 465), up to 80% of higher-order multiple births are considered to be due to ovarian stimulation and ART. Births resulting from infertility therapies account for around 1–3% of all singleton live births, 30–50% of twin births, and more than 75% of higher-order multiples (for review, see Ref. 3).

Pregnancy complications include increased risk of miscarriage, preeclampsia, growth retardation, and preterm delivery. Perinatal mortality rates are at least 4-fold higher in twin, and at least 6-fold higher in triplet births compared with singleton births (466). Moreover, the risks of prematurity in twin and higher-order multiple birth are increased 7- to 40-fold, and for low birth weight 10- to 75-fold, respectively. The incidence of child handicaps may be 50 and 100% higher in twins and triplets, respectively (467). Recent data generated from the national registry of Denmark suggest similar risk of neurological sequelae of twins from ART compared with both natural twins and ART singles (468). It has also been demonstrated recently that educational disadvantages related to low birth weight persist into early adulthood

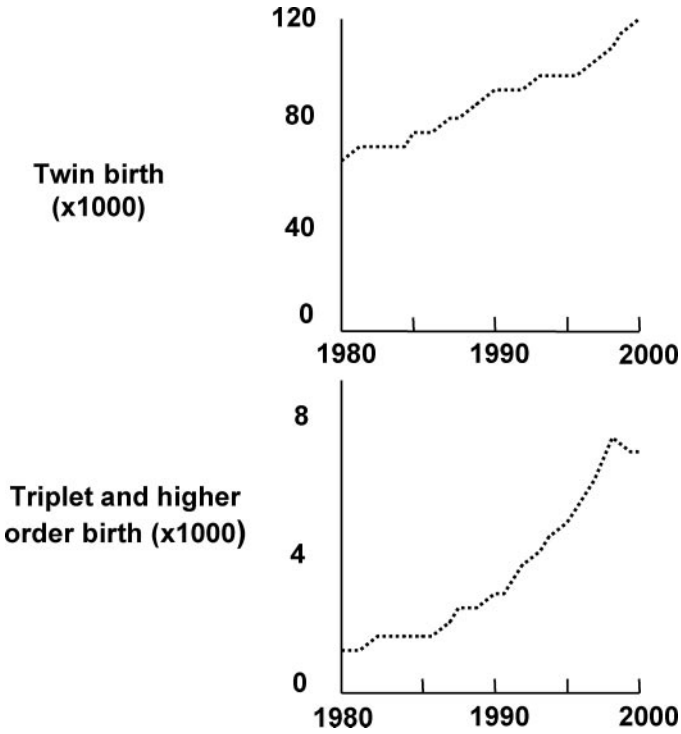

FIG. 6. Noted trends in multiple births in the United States for twin (*upper*) and triplet or higher order (*lower*) multiple births. [Reproduced from B. C. Fauser *et al.*: *Lancet* 365:1807–1816, 2005 (3) with permission from Elsevier].

(469). Higher-order multiple gestation is associated with significantly higher cesarean section rates, reduced gestational duration, lower birth weight, and increased perinatal mortality compared with twins (470–473). The association between (very) low infant birth weight and IVF is also clearly established (474), as is the impact of increasing numbers of multiple births on overall perinatal health (475).

Adverse outcomes among children conceived through IVF are largely associated with multiple gestation (475). However, a systemic review including 25 well-controlled studies established that even singleton pregnancies after IVF have worse outcomes (more preterm births, low birth weight, and admission to neonatal intensive care unit) compared with non-IVF singletons (476). A further, similar meta-analysis came to similar conclusions (477). Despite the reassuring low incidence of child abnormalities at birth, the monitoring of the safety of IVF with regard to rare congenital disorders or defective gene imprinting remains crucial (478).

4. Strategies for reducing the incidence of multiple birth after ART. A simple strategy to reduce multiple births would be to postpone ART in couples with a reasonable prognosis for spontaneous conception without intervention (3). In women with patent fallopian tubes, intrauterine insemination in spontaneous cycles may represent a preferable first line treatment because the risk of multiple pregnancy is not increased (3).

The chance of multiple pregnancy after IVF is directly related to the number of embryos transferred. A further strategy would therefore be to optimize access to IVF treatment to reduce the pressure to transfer multiple embryos. In

1998 in the United States, three or more embryos were transferred in 80% of IVF cycles, with four or more embryos in 47% of cycles. In contrast, in Europe three or more embryos were transferred in 51% of cycles and four or more in only 9%, although large differences between countries do exist (479). The policy of two-embryo transfer has been adopted by many major European IVF centers. Recently, it was demonstrated that in young women where two high-quality embryos are transferred, the chances of a twin pregnancy are actually higher than a singleton pregnancy (480) (Fig. 7**)**. An increasing number of leading centers in Europe are currently moving toward a policy of single-embryo transfer in selected women. The number of embryos transferred in the United States is substantially higher, with current revised guidelines still recommending the number for transfer to be between two and five, depending on patients' age and prognosis (481). In general, overall IVF results in Europe are (slightly) lower compared with the United States, but with an overall reduced incidence of multiple birth.

A further strategy would be to refine and standardize the reporting of treatment outcome, because patients tend to select the center with the "best" results (482). Research on less complex, more patient-friendly stimulation protocols (5) along with the transfer of a reduced number (preferably one) of embryos will only prosper in an environment where singleton healthy birth is considered the most appropriate endpoint of infertility treatment (483). This primary outcome needs to be judged in the context of the risk of adverse effects, complications, and costs per treatment (484–486) during a given period of time (487). The development of improved techniques to cryopreserve surplus embryos (with additional pregnancy chances in subsequent spontaneous cycles) is also crucial for the widespread acceptance of single-embryo transfer, because more good-quality embryos will be available for cryostorage. It appears that the use of single-embryo transfer in selected patients can significantly decrease overall twin pregnancy rates without reducing total pregnancy rates (3). If this phenomenon is confirmed, it will simplify counseling for single-embryo transfer.

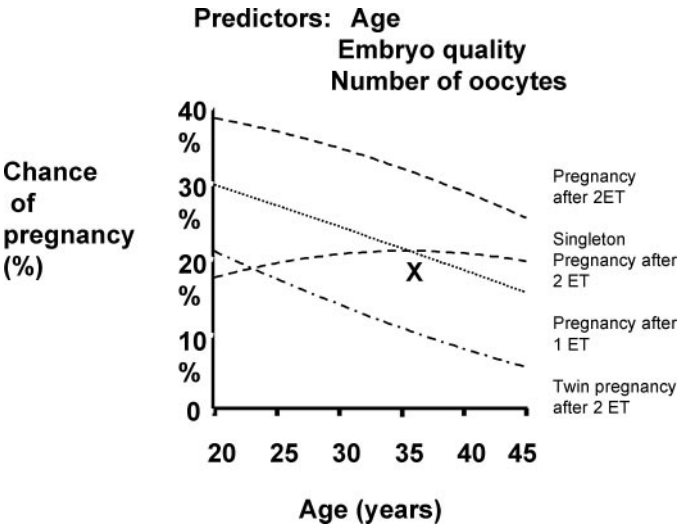

FIG. 7. Multivariate prediction (based on woman's age, quality score of embryos to be transferred, and number of oocytes obtained at retrieval) of the chance for a singleton or twin pregnancy should one or two embryos be transferred. In women older than 37 yr (*right of point X*), the chance of conceiving a singleton pregnancy is higher when two embryos are transferred rather than one. [Adapted from C. C. Hunault *et al.*: *Fertil Steril* 77:725–732, 2002 (480) with permission from the American Society for Reproductive Medicine.]

5. Long-term maternal risks associated with ovarian stimulation. Little reliable data exist regarding long-term health risks associated with the administration of gonadotropins, particularly in the context of IVF. Most published studies addressing this issue are flawed by low statistical power and lack of control for important confounders, such as presence and cause of infertility and type of fertility drug employed. In addition, follow-up periods are frequently short. This has led to inconsistent results and uncertainty regarding the safety of ovarian stimulation for IVF (488).

Recent large cohort follow-up studies linked to National Cancer Registries have, to date, shown no causative association between ovarian stimulation with exogenous gonadotropins and increased risk of malignant (488) or benign ovarian disease (489). However, microarray studies have demonstrated that gonadotropin stimulation may up-regulate oncogenes and tumor markers such as pleomorphic adenoma gene-like 1, tumor antigen L6, and claudin 3, while down-regulating certain suppression of tumorigenicity genes. At the same time, decreases in other specific tumor markers such as CD-24 antigen and pim-1 oncogene suggest that exogenous gonadotropins may also suppress specific cancers (490).

Before clear advice can be given to women undertaking IVF with ovarian stimulation, data from studies with longer follow-up periods in well-defined and well-characterized populations are required, with adequate controls for potential confounders.

VII. Contemporary Issues in Ovarian Hyperstimulation

A. Poor response to ovarian stimulation

Poor ovarian response to ovarian hyperstimulation for IVF is clearly associated with chronological aging. An age-related decline in response to stimulation with gonadotropins and a reduction in the number of oocytes retrieved (491), oocyte quality (492), fertilization rates (493), and ultimately embryo numbers (494) have been well documented. Many studies point to 40 yr of age as a significant cutoff for effectiveness of IVF (495–497). This age-related effect on pregnancy rates is similar to that reported in donor sperm programs (498) and chances for spontaneous pregnancy (145). A multiple regression analysis of factors influencing IVF outcomes revealed a predicted live birth rate of 17% per cycle at age 30, falling to just 7% at 40 yr and 2% at 45 yr of age (496). Although age is an important predictor of IVF outcome (150), chronological age is poorly correlated with ovarian aging (145). The concept of poor response as a feature of chronological and ovarian aging has been supported by recent studies linking poor response to ovarian hyperstimulation to subsequent early menopause (265, 499–502). In a study of normo-ovulatory women who had demonstrated a poor response to ovarian

stimulation for IVF, the number of antral follicles observed with ultrasound in a subsequent natural cycle was significantly lower than that in control subjects (503). Surprisingly, elevated follicular P concentrations were also observed in these women. Moreover, elevated baseline FSH levels were observed in less than 50% of the study patients, and inhibin B levels were normal in 80%. These findings support the concept that poor response to ovarian stimulation may represent the first clinical sign of ovarian aging, preceding the onset of endocrine changes or cycle abnormalities.

Poor response to ovarian stimulation is highly resistant to therapeutic intervention (265). Strategies for stimulating "low responders" include varying the dose or day of the cycle for initiating stimulation with gonadotropins. Studies undertaken so far have been unable to demonstrate a beneficial effect of gonadotropin dose increase in patients who exhibit a poor response to standard dose regimens (258, 265). Alternative approaches include early cessation or microdose GnRH agonist protocols. In one study in which GnRH agonist administration that commenced in the luteal phase was stopped at the onset of menses, one case of premature luteinization was observed in 200 cycles, and a reduced repeat cancellation rate was reported (504). Reducing the GnRH agonist dose in a microdose flare protocol also reduced the incidence of repeat cancellation for poor response without a concomitant increase in premature rises in LH or P (505). Although widely practiced, good evidence for the efficacy of these strategies is scarce. The use of GnRH antagonists in place of GnRH agonists has also been proposed on the premise of reduced suppression of endogenous gonadotropins. Large, well-designed studies to address this are still required.

In summary, application of these strategies may improve the chance of producing sufficient follicles to merit oocyte pickup, but none has been shown to improve pregnancy rates. In the absence of effective therapeutic strategies for poor response to ovarian stimulation, efforts have been made to develop means of predicting poor response to aid patient counseling. Markers of ovarian reserve discussed in *Section II* are employed to identify the patient for whom ovarian stimulation is unlikely to result in success. Although the number of antral follicles assessed by ultrasound may help identify older women with a better chance of responding to ovarian stimulation, it has recently been demonstrated that women predicted to have a poor response to stimulation are unlikely to benefit from a higher starting dose of gonadotropins in IVF (506–508). Hence, markers of ovarian ageing may be applied along with chronological age to identify patients with poor prognosis for successful IVF treatment. Under those circumstances, expectant management or IVF and oocyte donation may be advised.

B. Minimal vs. maximal ovarian stimulation

After the initial years of IVF, profound ovarian stimulation has been the rule for almost two decades. Stimulation of the growth of large numbers of follicles and the retrieval of many oocytes has been viewed as an acceptable marker of successful IVF treatment. Medication regimens to achieve profound ovarian stimulation are extremely complex and expensive and take many weeks of frequent injections and intense monitoring. Moreover, patient discomfort and chances for serious side effects and complications are considerable. In addition, this profound stimulation gives rise to greatly abnormal luteal phase endocrinology, and its impact on the chromosomal normality of embryos and endometrial receptivity and therefore IVF success is mostly unknown. Although current evidence suggests that the cotreatment with GnRH antagonist compared with the agonist may slightly reduce chances for success, from the perspective of patient convenience, and allowing for further refinements in its application, it seems justified to predict that the GnRH antagonist will eventually replace the agonist (243).

Current attitudes to profound ovarian stimulation should change (3), certainly with the growing tendency currently toward the transfer of a reduced number of embryos. Emphasis may now be directed toward the development of mild stimulation protocols (393, 509) or the improvement of natural cycle IVF outcomes (510). Improvements in the efficiency of cryopreservation programs will be of paramount importance, allowing women the additional chance of pregnancy without going through ovarian stimulation and follicle puncture.

Previous studies in normo-ovulatory female volunteers (511, 512) confirmed that the development of multiple dominant follicles can be induced by interfering with decremental FSH concentrations during the mid- to late-follicular phase. These observations are in agreement with previous findings in the monkey model (513, 514). In a randomized study, almost all the pregnancies occurring after mild stimulation were observed in patients with a low oocyte yield, whereas no pregnancies were observed when a similar yield was obtained after conventional IVF (336) (Fig. 8**)**. These data clearly suggest that the relationship between oocyte quality and quantity of oocytes retrieved is dependent on the applied stimulation regimen and that a low response to maximal stimulation (suggestive of ovarian aging) is distinctly different from normal response to low stimulation.

C. hCG substitutes for inducing final oocyte maturation

In the natural normo-ovulatory cycle, rupture of the dominant follicle and release of the oocyte are triggered by the midcycle surge of LH. This sudden enhancement of pituitary synthesis and release of LH (and FSH) is elicited by high late-follicular phase E_2 levels in combination with slightly elevated P levels (515). In stimulated cycles for IVF, estrogen levels are prematurely elevated, inducing unpredictable but advanced LH rises. As mentioned before, GnRH analog cotreatment is required to prevent this from happening. Consequently, exogenous hCG is used during the late follicular phase under these circumstances to replace the endogenous LH surge. This approach has been considered the standard of care for the induction of the final stages of oocyte maturation before oocyte retrieval along with corpus luteum formation in IVF (516). Exogenous hCG is also implicated in sustained luteotropic activity (317) due to its prolonged circulating half-life (517). Unfortunately, hCG is therefore also believed to contribute to chances of developing the dangerous OHSS (386).

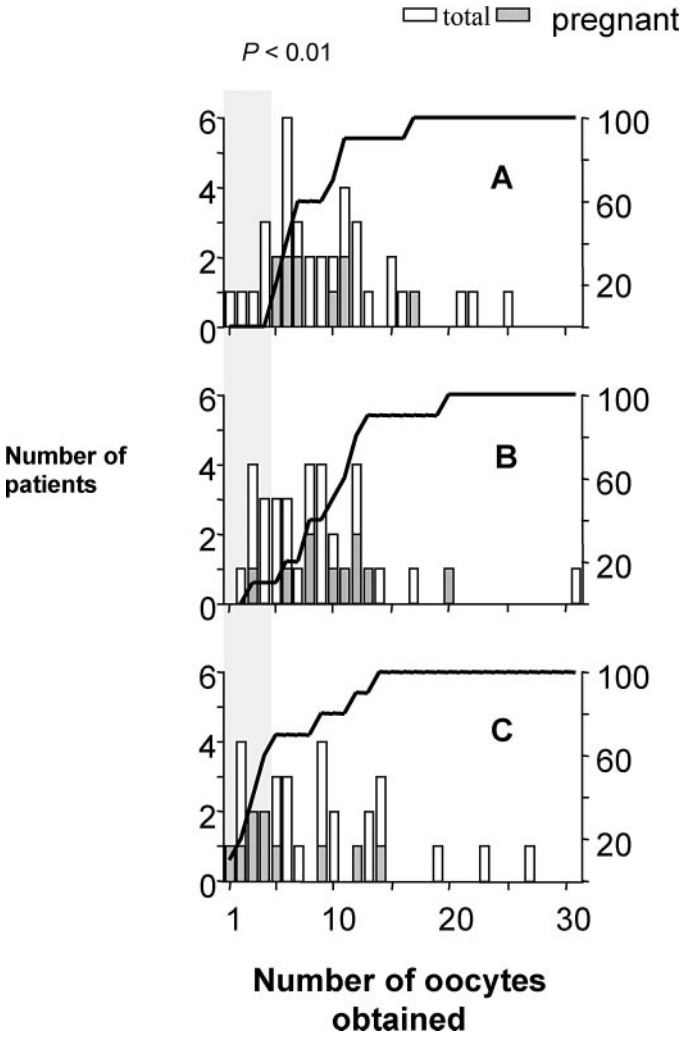

FIG. 8. Number of women undergoing IVF who did or did not achieve a pregnancy in relation to the amount of oocytes retrieved, comparing conventional hyperstimulation with a GnRH agonist long protocol (A) with two mild stimulation protocols employing GnRH antagonist cotreatment (B and C). [Adapted with permission from F. P. Hohmann *et al.*: *J Clin Endocrinol Metab* 88:166–173, 2003 (336). © The Endocrine Society.]

Initial studies during ovarian stimulation for IVF (before the widespread use of GnRH agonist cotreatment) showed that an endogenous LH surge could be induced reliably at midcycle by the administration of native GnRH or a bolus injection of GnRH agonist (518, 519). The induction of an endogenous LH (and FSH) surge is more physiological compared with exogenous hCG due to the much shorter half-life (72, 520). Moreover, luteal phase steroid concentrations seem closer to the physiological range (71), which may improve endometrial receptivity (521).

Because the follicular phase cotreatment with GnRH agonist has been the standard of care for over a decade, alternative approaches for the induction of oocyte maturation have received little attention in recent years. However, the suppressive effect of follicular phase GnRH antagonist administration can be reversed immediately by administering native GnRH or GnRH agonist (31, 389). Indeed, a recent randomized trial could confirm that the triggering of final stages of oocyte maturation can be induced effectively by a single bolus injection of GnRH agonist, even after the follicular phase cotreatment with a GnRH antagonist. This was demonstrated by the observed gonadotropin surge and quality and fertilization rate of recovered oocytes (71). Whereas high rates of fertilization have been observed in oocytes obtained after GnRH agonist triggering in this way, a recent study has revealed low implantation rates after embryo transfer. Although this finding may have reflected inadequate luteal support, further work is required before GnRH agonists can be introduced into clinical practice for this indication (522).

recLH and rechCG recently became available for clinical use. It was demonstrated in a large randomized trial comparing 250 mg rechCG *vs.* 5,000 IU uhCG for the induction of oocyte maturation in a total of 190 women undergoing IVF that the number of mature oocytes retrieved, along with luteal phase serum P and hCG concentrations were significantly higher after administration of rechCG (218). However, a recent meta-analysis revealed no differences in efficacy between rechCG and uhCG when used to trigger final oocyte maturation in IVF (219). rechCG compared with uhCG shows equivalent efficacy in ovulation induction in World Health Organization Group II anovulatory infertility (218). In a comparison of two dose regimens, 250 mg rechCG was found to be as effective as a 500-mg dose (523). With regard to recLH, a minimal effective dose of 15,000–30,000 IU compared with 5,000 IU uhCG in IVF patients showed a similar number of oocytes, embryos, and clinical outcomes (217). Considering the short half-life of recLH, two injections with a 1- to 3-d interval may be considered.

D. Chromosomal competence of embryos

In the early 1990s, the development of fluorescent *in situ* hybridization (FISH) technology enabled the chromosomal constitution of a single cell from arrested human embryos to be analyzed. Classic cytogenetic methods had previously indicated that between 23 and 80% of embryos are aneuploid (524). The use of FISH provided more information as to the types of chromosomal abnormalities and how they arise. Reported proportions of chromosomal abnormal preimplantation embryos range between 30 and 70% in embryos at d 2/3 of development (525, 526). Aneuploidy rates appear to increase with age (527). In women over 44 yr old, the aneuploidy rate rises to more than 95% (528). The high rate of aneuploidy encountered in embryos arising from IVF may provide some explanation for the failure to significantly increase implantation rates in IVF. Appreciation of this, and of the observation that at least 50–60% of spontaneous miscarriages from clinically recognized pregnancies have abnormal chromosomal compliment (529), has led to the institution of preimplantation genetic screening (PGS) for aneuploidy in an attempt to improve outcomes from IVF. As new technology has been introduced, PGS has revealed higher rates of aneuploidy in IVF embryos. PGS is now offered to couples undergoing IVF to improve embryo selection and pregnancy rates and reduce the risk of miscarriage due to aneuploidy. However, data from the few randomized controlled studies

available have failed to demonstrate any clinical impact of PGS on IVF outcomes or in reducing miscarriage rates (528, 530). The limited number of chromosomes analyzed using FISH techniques, possible damage to the embryo due to the removal of one or two blastomeres for analysis, and errors in hybridization and interpretation may partially account for this. However, it is now clear that the phenomenon of chromosomal mosaicism in preimplantation embryos complicates interpretation of PGS (531) and thus the identification of embryos that will develop normally. In human preimplantation embryos, especially those with abnormal morphology, chromosomal mosaicism has been shown to be a normal feature (525, 532–535). The data reported appear to depend on the number of probes applied simultaneously, the type of probes used, embryo morphology, embryo development, and the presence of multinucleated blastomeres. In total, at least 29% of morphologically normal embryos are chromosomally abnormal (532) at the cleavage stages. In human blastocysts chromosomal mosaicism was reported in 29% (536). Others (537, 538) reported that during preimplantation development, the percentage of embryos showing chromosomal mosaicism increases to almost 100% at the blastocyst stage. The percentage of abnormal cells per embryo was 16%. Studies applying the comparative genome hybridization technique, in which all chromosomes are assessed, reported a similar percentage of abnormal cells in the embryo (539, 540). PGS has been shown to be an invaluable research tool in increasing understanding of factors involved in determining embryo quality. Recently, PGS has been employed to study the impact of conventional *vs.* mild stimulation regimens on aneuploidy rates in the resultant embryos (442). These were shown to be reduced after mild ovarian stimulation. Beyond research applications, the role of PGS and comparative genome hybridization as a clinical tool in IVF remains to be defined in well-designed randomized controlled studies.

VIII. Conclusions and Future Perspectives

Current IVF practice has its roots in the pioneering research carried out in the 20th century into ovarian physiology; the isolation, purification, and production of gonadotropins; as well as the ground-breaking work by Edwards and others which led to the birth of Louise Brown. Much has been learned in the past 25 yr regarding the mode of action and effects of ovarian stimulation for IVF. At the beginning of the second "IVF century" we are in a position to critically evaluate the paradigms of ovarian stimulation developed in the early days, which are still widely applied today. The field has now matured to the extent in which the focus is not purely on increasing surrogate outcomes such as conception rates, although this remains a challenge. Increasingly, IVF is being viewed in the context of long-term health outcomes for women and their offspring and cost effectiveness and as a tool for prevention of morbidity, as exemplified by preimplantation genetic screening and diagnosis.

Moreover, the development of new molecular tools in the fields of genomics, proteomics, and pharmacogenomics are providing new windows on ovarian and endometrial physiology and the impact at the molecular level of stimulation regimens. Although these novel techniques will have a major impact on our knowledge of ovarian stimulation, it is clear that much remains to be learned about the endocrinology of follicle development, oocyte maturation, and ovulation. Recent studies indicate that LH has an important role in preovulatory follicular development. Increasing understanding of the role of individual gonadotropins, combined with the availability of pure recombinant preparations of LH, FSH, and hCG open the way to a more sophisticated and individualized approach to ovarian stimulation.

Powerful molecular modeling techniques are now being employed to develop chemical gonadotropin receptor agonists that mimic the effect of the hormonal ligands. These compounds may be active in oral form. These developments promise to further reduce the physical burden that IVF treatment presently constitutes for women faced with subfertility.

IVM of oocytes has been proposed as a means of obtaining gametes for IVF without exposing the patients to the potential risks of ovarian stimulation with gonadotropins. The ability of immature oocytes to resume meiosis spontaneously when removed from the follicle was first demonstrated in 1935 (541), and this was later confirmed by Edwards (542), who subsequently demonstrated the fertilization of IVM human oocytes (543). Initial reports of birth after IVM were in from immature oocytes derived from stimulated cycles (544). Ten years later, a live birth was reported after IVM using immature oocytes recovered by puncture during nonstimulated cycles in a woman with PCOS (545). Because ovarian stimulation is associated with a high risk of complications such as OHSS in PCOS, this group of patients might be considered to particularly benefit from IVM in IVF.

Implantation rates of embryos derived from IVM in the nonstimulated cycle are low (546). Priming with FSH for 2–3 d to stimulate the follicle to develop to 8- to 12-mm diameter has been associated with improvements in implantation rates (547). Additional treatment with 10,000 IU hCG 36 h before oocytes retrieval was also shown to improve maturation rate of immature oocytes (548) and accelerate the maturation process (337). However, even with such intervention, pregnancy rates of 30% have only been obtained by transplanting multiple embryos, because implantation rates remain 10–15%. Insufficient data are currently available from follow-up studies to assess the safety of this technique for offspring (298).

Mild stimulation regimens in combination with single-embryo transfer may offer the optimal combination of effective, cost-effective treatment that minimizes side effects and the morbidity and mortality associated with multiple pregnancy. There is an increasing consensus that multiple pregnancy arising from IVF represents the major clinical problem to be addressed. Strategies for prevention, outlined in this article, are clear. However, implementing these in the current commercially competitive context in which IVF is practiced is more challenging.

Crucial to the success of implementing strategies such as single-embryo transfer on wide scale will be getting agreement as to how success in IVF should be defined. The current focus on pregnancy rates per cycle encourages maximal stimulation and transfer of multiple embryos. If live singleton

birth per started treatment (rather than per cycle) became adopted as the measure of care by the institutions that govern IVF throughout the world, and if those who publish and compare outcomes from different centers, then a major step would be taken toward reducing the burden of IVF in the couple, their offspring, and society.

Acknowledgments

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N.S.M. and B.C.J.M.F. have received lecture fees and research grants from Serono, Organon, and Ferring. L.C.G. has served as a consultant to Organon. R.L.S. has no potential conflicts of interest.

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