Manipulation of Human Ovarian Function: Physiological Concepts and Clinical Consequences*

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I. Introduction

IN RECENT years much new information related to regulation of human follicle development has become available. Recent techniques for the investigation of human ovarian tissue include immunocytochemistry (allowing direct visualization of proteins in tissue), or *in situ* hybridization for the *in situ* detection of DNA or RNA. New tools such as pelvic ultrasound have been developed allowing the longitudinal monitoring of follicle growth dynamics in a given patient. In addition, assays of steroids and peptides in serum and follicle fluid, together with *in vitro* cultures of human ovarian cells, have generated additional information regarding endocrine and para-/autocrine factors regulating follicle growth.

New insight in the interplay between systemic and intraovarian factors regulating development and atresia of follicles may have significant implications. Relevant clinical conditions include ovarian ageing as well as chronic anovulation in patients presenting with serum FSH and estradiol (E_2) hormone levels within the normal range, frequently diagnosed as polycystic ovary syndrome (PCOS). More effective and safe protocols for stimulation of ovarian function for infertility therapy may be developed. This involves both gonadotropin induction of ovulation (aiming at single dominant follicle growth in anovulatory patients) and so-called 'controlled' ovarian hyperstimulation for *in vitro* fertilization (IVF) (aiming at interfering with single dominant follicle selection to induce ongoing multiple follicle development in ovulatory women).

Due to ongoing concern regarding the potential for side effects and long-term health hazards, doses of combined estrogen/progestin steroid contraceptive pills have been decreased continuously since their introduction in the 1960s. It has been noticed subsequently that tolerance for omission of pill intake, especially around the pill-free interval, has diminished substantially in women using regimens presently on the market. Modest suppression of pituitary gonadotropin secretion during pill intake and recovery of FSH release during the pill-free week creates a situation resembling the early follicular phase of the normal menstrual cycle and allows for substantial residual ovarian activity.

Concepts involved in regulation of follicle growth during gonadotropin induction of ovulation (attempting to enhance fertility) as well as during steroid contraception (aiming at inhibiting fertility) are derived from recent findings regarding regulation of ovarian function under physiological circumstances. Therefore, these three conditions have been selected as the major focus of the present review.

II. Dynamics of Normal Human Follicle Growth and Selection

A. Gonadotropin-independent and -dependent follicle growth

Resting primordial follicles continuously enter the growing pool throughout life (for review see Refs. 1–3). The magnitude of depletion of the primordial follicle pool is dependent on age and is most pronounced during fetal development. Oocytes are detectable in fetal ovaries after 16 weeks of gestational age. The great majority of oocytes are lost after the fifth month of intrauterine life, when a maximum of approximately 7 million germ cells have been reported (3). The presence of growing follicles in fetal ovaries has been substantiated extensively (4). At birth, both ovaries contain approximately 1 million primordial follicles. Reproductive life starts with approximately 0.5 million primordial follicles at menarche. Thereafter, loss of follicles takes place at a fixed rate of around 1000 per month, accelerating beyond the age of 35 (5–8). Studies in the rat model suggest indeed that follicle loss is inversely related to the number of primordial follicles present in the ovaries (9). Once follicles are stimulated to grow, they can either reach full maturation and ovulate or become atretic. Follicles are present in the ovary at different stages of development, and large numbers of follicles of different sizes can be observed at any given point of the menstrual cycle (10). The distribution of developmental stages of follicles entering atresia may vary with age (11). It is generally believed that, especially at an early age, loss of follicles is largely due to atresia of primordial follicles (12). It is unknown as yet which factors regulate initiation of growth of primordial follicle (12, 13) and whether maturing follicles may enter atresia at all developmental stages (14).

When primordial follicles enter the growth phase they enlarge by an increase in size of the oocyte together with granulosa cell proliferation (primary follicle). Transition into the secondary follicle stage involves alignment of stroma around the basal lamina and the development of an independent blood supply. The stroma subsequently differentiates into a theca externa (similar to surrounding stroma cells) and a theca interna layer. Theca interna cells express LH receptors early on (15). Development of an antral cavity (at a follicle size ~100 to 200 μm) divides granulosa cells in cells surrounding the oocyte (cumulus) and cells that border the basement membrane. During early preantral follicle development, FSH receptors also become detectable on granulosa cells (7, 15, 16). The time span between a primary and an early antral follicle in the human is unknown but is proposed to be several months. Subsequent stages from early antral to preovulatory follicles exhibit clear morphological characteristics, and the time interval is assessed to be approximately

3 months (for review see Ref. 12) (Fig. 1). An increase in the number of granulosa cells is critically important for the advancement in developmental stages of the follicle. The time interval required for a given follicle to pass these different developmental stages can therefore also be assessed by calculating the granulosa cell-doubling time (duration of mitotic activity *in vitro*) (17).

Under normal conditions, only about 400 follicles reach the mature preovulatory stage and ovulate in a lifetime. Hence, loss of follicles due to atresia — with apoptosis [*i.e.* programmed cell death (18)] as the underlying cellular mechanism — rather than growth and subsequent ovulation should be considered the normal fate of follicles. The importance of oxidative stress in inducing atresia (19) and gonadotropins and various growth factors ('survival factors') to suppress apoptosis (20, 21) has been emphasized recently. FSH decreases apoptosis in granulosa cells obtained from hypophysectomized rats (22) and prevents apoptotic changes of cultured preovulatory follicles (23).

In the human the process of initiation of follicle growth and subsequent exhaustion of the resting pool of primordial follicles appears to be regulated independently of stimulation by gonadotropins (24). Follicles become dependent on stimulation by FSH only at an advanced developmental stage, as will be discussed later (*Section II.E*). For instance, follicles grow up to the early antral stage in long-term hypophysectomized animals (25, 26). Similar numbers of maturing follicles, as compared with controls, have been found in anencephalic fetuses (27, 28), and exposure of ovaries to high gonadotropin levels has failed to result in accelerated follicle loss (12). It appears in the human that follicle development up to the antral stage continues throughout life until depletion of follicles around menopause, even under conditions in which endogenous gonadotropin release is diminished substantially (5, 29). Such conditions include prepubertal childhood (30–33), pregnancy (34–37), and the use of steroid contraceptives (see *Section IV*). In addition, follicle growth up to the early antral stage has been described in women with absent gonadotropin secretion, either due to hypophysectomy, as discussed by Block (1), or to hypothalamic/pituitary failure (38). However, observations in hypogonadal mice suggest that gonadotropins do play a role in initiation and continuation of follicle growth (39). In the rat model it has been suggested that theca cell differentiation and early preantral follicle growth is dependent on subtle stimulation by LH (40, 41). In addition, assessment of ovarian morphology of term infant monkeys showed a reduced number of primordial and primary follicles and increased follicle atresia after hypophysectomy (42). In conclusion, the question of whether the extent and rate of early follicle growth is dependent on exposure to minute amounts of gonadotropins remains unsolved (43, 44). Improved knowledge regarding mechanisms regulating initiation of primordial follicle growth as well as atresia of early stages of follicle development may shed more light on clinical conditions such as ovarian ageing and premature ovarian failure, as well as the great individual variability in menopausal age.

In contrast to early follicle development, stimulation by FSH is an absolute requirement for development of large antral preovulatory follicles. Duration and magnitude of FSH
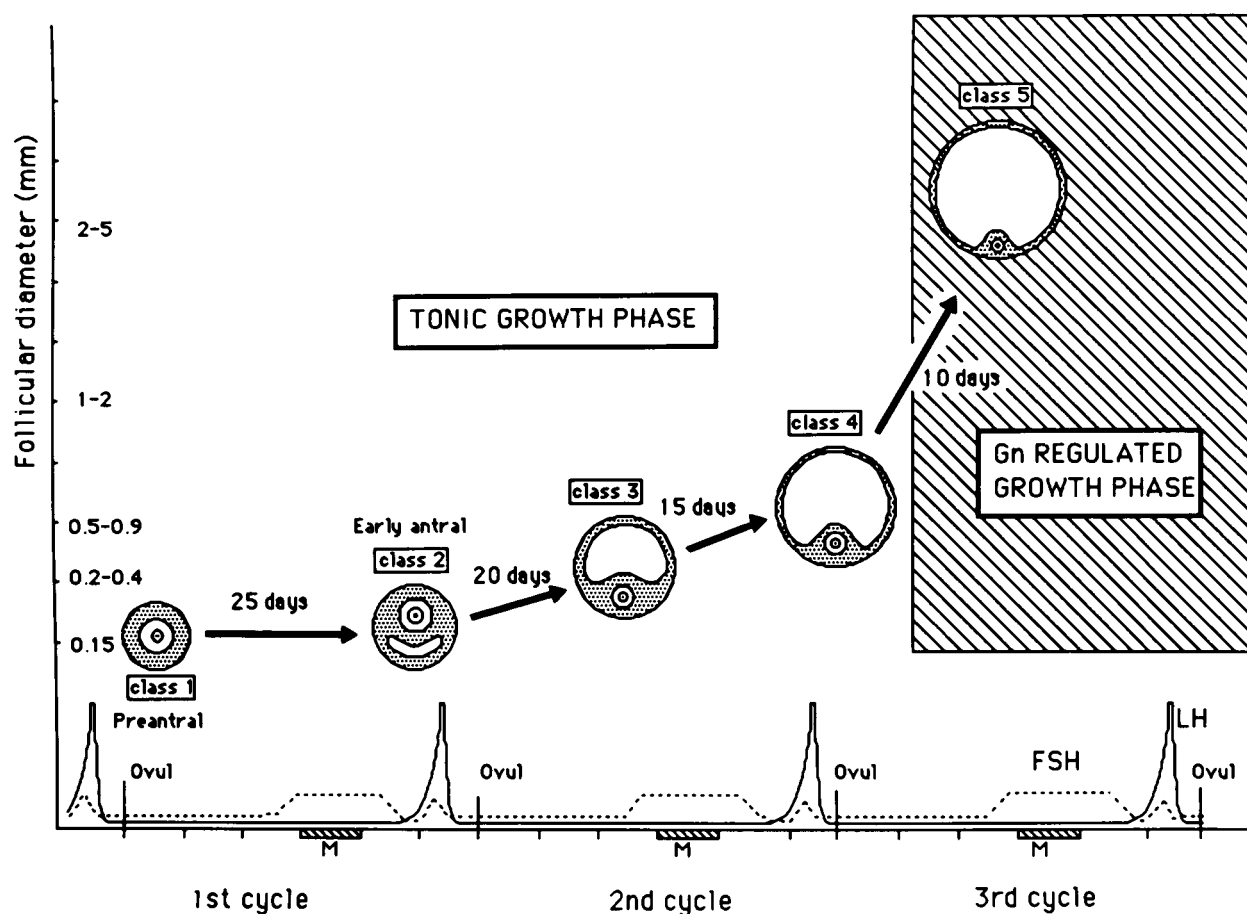
FIG. 1. Schematic representation of human ovarian follicle development. Primordial follicles entering the growth phase form primary follicles (class 1). This is followed by gonadotropin-independent (tonic) growth (class 1 to 4), and eventually gonadotropin (Gn)-dependent growth. Note that the overall development from a class 1 to a class 5 follicle takes three cycles [Reproduced with permission from A. Gougeon].

stimulation will determine the number of follicles with augmented aromatase enzyme activity and subsequent E_2 biosynthesis. High FSH levels usually occurring during the luteo-follicular transition give rise to continued growth of a limited number (cohort) of follicles. Subsequent development of this cohort during the follicular phase becomes dependent on continued stimulation by gonadotropins. In contrast to other primate species such as the Booroola sheep (14, 45), in the human only a single follicle from the cohort is selected to gain dominance and ovulate every cycle. Remaining cohort follicles enter atresia due to insufficient support by reduced FSH levels. The only exception to this rule is familial dizygotic twins in which ongoing growth and ovulation of multiple follicles occur (46, 47). A reduced rate of follicle atresia due to altered intrafollicular steroidogenesis independent from gonadotropins has recently been proposed as the underlying cause (48).

B. Intrafollicular endocrine changes

The majority of enzymes involved in the biosynthesis of ovarian steroids belong to the cytochrome P-450 gene family (for review see Refs. 49 and 50). This group of enzymes includes: 1) Cholesterol side-chain cleavage enzymes (P-450SCC), which convert cholesterol to pregnenolone. 2) The

P-450C17 enzyme (involving both 17α-hydroxylase and C17,20-lyase activity) converts both progestins (pregnenolone and progesterone) to androgens [dihydroepiandrosterone and androstenedione (AD), respectively]. 3) The aromatase enzyme complex (P-450A ROM), converts androgens [AD and testosterone (T)] to estrogens (estrone and E_2, respectively). Moreover, a specific DNA sequence, termed Ad4, has recently been identified as a transcription factor regulating the expression of steroidogenic P450 genes. The expression of Ad4-binding protein (a zinc finger DNA-binding protein also known as steroidogenic factor-1) has been shown to correlate with the immunolocalization of steroidogenic enzymes in the human ovary (51).

Two enzymes that are not members of the P-450 gene family are also important for gonadal steroid synthesis: 3β-hydroxysteroid dehydrogenase, converting Δ5-steroids (such as pregnenolone) to Δ4-steroids (such as progesterone), and 17 ketosteroid reductase converting AD to T and estrone to E_2.

The cholesterol side-chain cleavage enzyme represents the major rate-limiting step in steroid hormone synthesis. Moreover, proteins involved in the acquisition of cholesterol (including lipoprotein receptors and enzymes involved in *de novo* cholesterol synthesis) have also been shown to be im-

portant for sufficient steroid biosynthesis (50). Patients have been described with mutations in DNA encoding for a protein involved in cholesterol transport within the cell (so-called steroid acute regulatory protein) (52) or encoding for specific enzymes involved in the steroid synthesis pathway (for review see Ref. 53). The significance of each step for normal steroid biosynthesis and subsequent ovarian function has been clarified by the careful description of underlying gene abnormalities and the phenotype expression in the event that certain steroids are lacking.

In vitro studies using cells isolated from human ovarian follicles have demonstrated convincingly that theca cells are the source of follicular androgens (54, 55) — predominantly AD (56, 57) — whereas granulosa cells only produce E_2 when androgens are added to the culture medium (58–60). In the human ovarian follicle, immunocytochemistry (with the use of antibodies against specific enzymes, allowing direct visualization of the distribution of the enzyme in tissue) as well as Northern blot analysis of RNA has shown the P-450C17 enzyme to be restricted to the theca cell layer (61, 62), consistent with the notion that these cells are the major site of intrafollicular androgen production. mRNA levels for P-450C17 are increased dramatically in preovulatory follicles (63), which correlate well with augmented 17α-hydroxylase activity of human theca cells in culture (64). Small antral follicles were shown to lack P-450AROM mRNA. However, appreciable quantities of mRNA (63, 65, 66) and the aromatase enzyme (62, 67) were observed in dominant follicles in the late follicular phase. These observations are in keeping with the high level of aromatase enzyme activity expressed *in vitro* by granulosa cells obtained from preovulatory follicles (59, 68). In addition, mRNA expression is in good agreement with immunolocalization of the aromatase enzyme (66). Synthesis of the P-450AROM enzyme could also be induced by FSH administration to human granulosa cells in culture (69). When follicles mature, granulosa cells also exhibit elevated mRNA levels for P-450SCC, LH receptor, activin, and inhibin (70).

The theca interna layer of developing follicles responds to LH and synthesizes androgens (71, 72). AD and its immediate metabolite T are transferred from the theca layer to the intrafollicular compartment. For this reason these steroids are present in large quantities in ovarian follicles of all sizes and represent the main steroid produced by early antral follicles (73–75). Atretic follicles of all sizes (between 2 and 13 mm diameter) also contain high androgen levels (57, 76) and low E_2 concentrations (77). Granulosa cells become responsive to FSH only at more advanced stages of development and are capable of converting the theca cell-derived substrate AD to E_2 by induction of the aromatase enzyme. This so-called 'two-gonadotropin, two-cell' concept emphasizes that adequate stimulation of both theca cells by LH and granulosa cells by FSH is required for adequate E_2 biosynthesis, as has been recognized since the 1940s (54, 78–82).

Large (>8 mm diameter) follicles in the mid- and late follicular phase of the menstrual cycle contain appreciable (up to 10,000-fold) higher quantities of E_2 compared with small follicles, as has been shown by numerous authors (60, 75, 76, 83–87). Intrafollicular E_2 concentrations were up to 40,000-fold higher than those in peripheral plasma, and 20-fold higher concentrations of E_2 have been observed in venous blood draining the ovary containing the dominant follicle as compared with the contralateral side (88, 89). It has been demonstrated in IVF patients that a correlation exists between the E_2/androgen ratio in follicle fluid and follicular health and fertility potential of oocytes (90). After enucleation of the largest follicle no further differences were found in steroid levels in blood draining both ovaries (91). A correlation between intrafollicular E_2 concentrations and follicle diameter has been substantiated in large dominant follicles (75, 77, 83). All studies show low E_2 levels in relatively small (<10 mm diameter) nondominant follicles (57, 68, 76, 77, 83), and the absence of a correlation between follicle size and E_2 levels in this size range (Fig. 2) was emphasized recently (75). The magnitude of E_2 synthesized by granulosa cells *in vitro* is dependent on the size of the follicle from which cells were obtained, with AD metabolized to E_2 only by granulosa cells from follicles beyond 8–10 mm in diameter (59, 68, 92). Follicle fluid E_2 concentrations are also correlated with the amount of aromatase activity expressed *in vitro* (60). In addition, granulosa cells in culture produce larger quantities of E_2 in response to similar doses of FSH if cells were obtained from larger (>8 mm) follicles (59, 68, 92), suggesting increased sensitivity. Moreover, lower doses of FSH induce similar E_2 production by cultured rat granulosa cells obtained from larger follicles, again indicating that cells obtained from more mature follicles exhibit augmented sensitivity for stimulation by FSH (93). Finally, a distinct relationship was observed between follicle diameter and the number of granulosa cells that was recovered at each size (94).

Collectively, overwhelming *in vivo* and *in vitro* evidence, both in animal models and in the human, suggest that enhanced E_2 biosynthesis is closely linked to preovulatory follicle development and that high estrogen output of the dominant follicle is regulated by FSH-stimulated granulosa cell function. Development of smaller follicles in the early follicular phase, although dependent on FSH, is not associated with increased E_2 production.

C. Are estrogens needed for follicle development?

As discussed above, dominant follicle development in the human is closely associated with increased follicular estrogen biosynthesis. E_2 receptors have been shown to be present in rat granulosa cells, as studied by ligand-binding assays (95). Numerous *in vitro* studies have shown for the rat model that E_2 plays important autocrine roles in stimulating FSH-induced granulosa cell proliferation (76, 96), aromatase enzyme induction (97–99), production of inhibin (100), increase in E_2 and FSH receptors (101), and formation of LH receptors on granulosa cells (102, 103). In addition, E_2 exhibits a paracrine action on adjacent theca cells by inhibiting androgen production (72). Estrogen (diethylstilbestrol) treatment of immature hypophysectomized rats stimulates growth of large numbers of follicles. Human chorionic gonadotropin (hCG) and FSH-induced follicle development could be inhibited by the administration of estradiol antiserum (104), suggesting again autocrine stimulatory roles for endogenous estrogens. Estrogens have also been shown to inhibit apo-
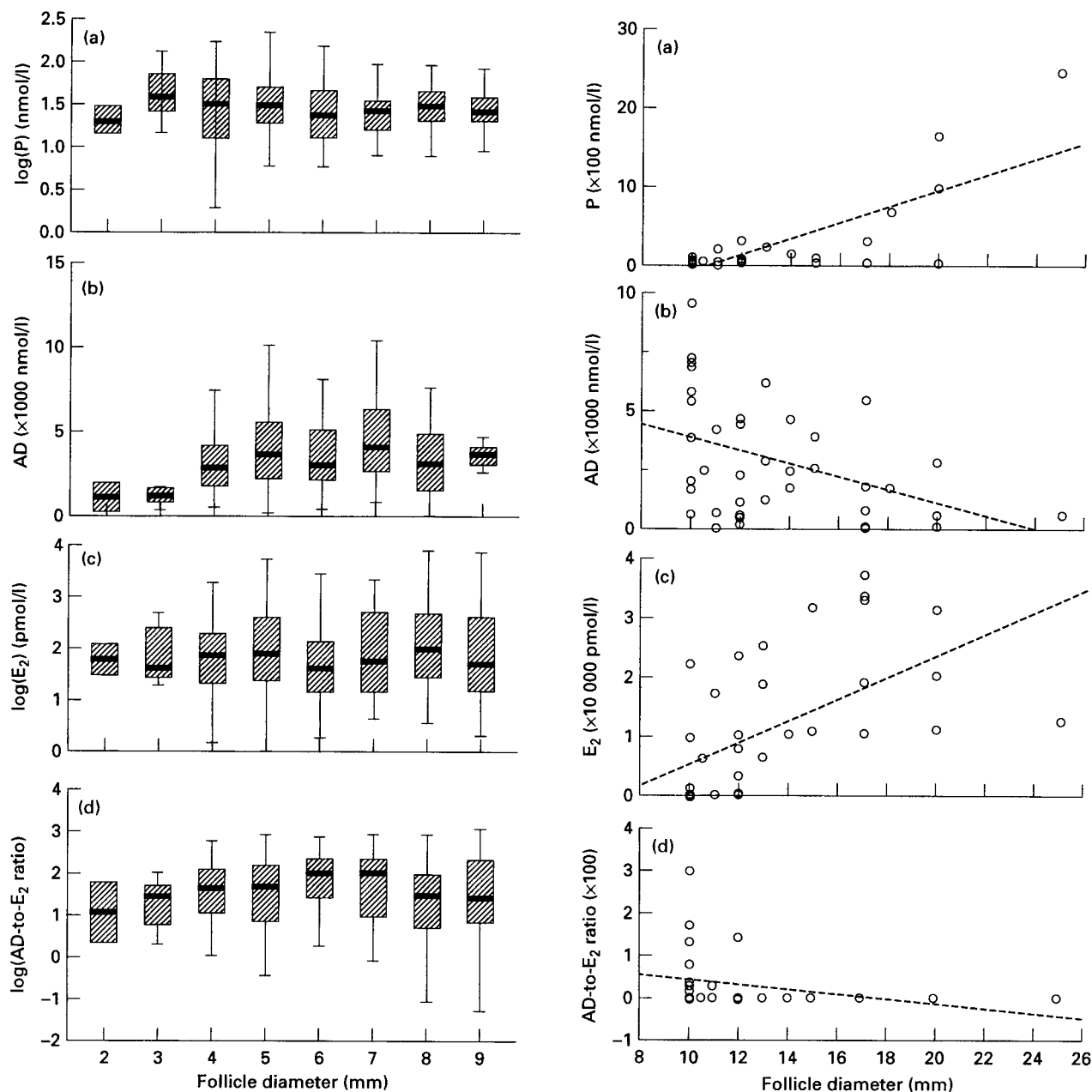
FIG. 2. Intrafollicular steroid concentrations as related to follicle diameter in 281 nondominant follicles punctured during various phases of the menstrual cycle (*box and whisker plots; left panel*), and 45 dominant follicles punctured during the late-follicular phase (*right panel*) obtained from 55 regularly cycling volunteers. Please note that follicle size is only associated with intrafollicular E_2 levels when a diameter of 10 mm or more is obtained. P, Progesterone; AD, androstenedione; E_2, estradiol. [Reproduced with permission from T. van Dessel *et al.*: *Clin Endocrinol (Oxf)* 44:191–198, 1996 (75).]

ptotic changes of ovarian follicles (20). Based on these observations, the concept has arisen that augmented intrafollicular E_2 production is a *conditio sine qua non* for ongoing follicle maturation. In fact, absent induction of aromatase enzyme activity has been widely accepted as the underlying cause of follicle maturation arrest and subsequent anovulation in PCOS (105).

Several lines of evidence, however, gave strong support to the notion that this may not be the case for higher species, including the human. Under normal conditions, augmented E_2 levels may merely be associated with normal follicle de-

velopment. A deficiency of the 17α-hydroxylase enzyme due to a specific gene defect affects both adrenal steroidogenesis and androgen and estrogen production by the ovary. This condition is characterized by hypergonadotropic hypoestrogenic primary amenorrhea, with arrest of follicle development at the early antral stage (106). However, normal follicle development could be induced in these patients by FSH treatment for IVF (after GnRH agonist suppression of endogenous gonadotropin release) despite extremely low intrafollicular levels of AD, T, and E_2. Oocytes could be obtained and fertilized *in vitro* resulting in normal early embryo

development (107, 108). In another patient suffering from a partial P-450C17 (17, 20-lyase step) deficiency, follicle growth could also be achieved after the administration of exogenous FSH despite low intrafollicular E_2 levels (109). Subsequent IVF and cleavage rates were not different from normal. Moreover, two unrelated females have been described recently with mutations in the CYP19 gene (consisting of 10 exons, and localized on chromosome 15, q21.1 region), resulting in the total absence of aromatase enzyme activity (110, 111). Large ovarian cysts have been described in both patients, suggesting that growth of antral follicles can occur in the absence of intraovarian estrogen biosynthesis. Recent experiments in monkeys treated with an aromatase inhibitor between day 8 and 10 of the follicular phase have also excluded the possibility that increased levels of circulating E_2 in the late follicular phase is required to sustain follicle maturation (112).

We have recently participated in a study on safety and pharmacokinetic properties of human recombinant FSH (113, 114) in hypogonadotropic female volunteers. The complete absence of endogenous as well as exogenous LH in these subjects did provide the unique opportunity to study effects of FSH alone on ovarian steroid production and fol-

licle growth (115). Despite a significant increase in serum FSH levels, in the same order of magnitude as the intercycle rise in FSH during the normal menstrual cycle, serum E_2 levels remained low. However, development of multiple preovulatory follicles emerged within 14 days. In a single subject, three large follicles between 13 and 18 mm in diameter were aspirated, and extremely low intrafollicular levels of AD and E_2 were found (Fig. 3) (87). A normal rise in immunoreactive serum inhibin levels in the majority of these women excluded the possibility of granulosa cell abnormalities *per se* (38). A discrepancy between serum E_2 levels and follicle development has also been observed in hypogonadotropic women comparing purified FSH of urinary origin and human menopausal gonadotropin (HMG; 1:1 ratio of LH to FSH activity) (116). When urinary FSH was combined with long-term GnRH agonist comedication suppressing the endogenous release of LH and FSH, similar observations were reported (117). It is of special interest to note that large antral follicles were also observed in the ovaries of two amenorrheic patients described with inactivating mutations of the LH receptor (and consequently low E_2 production) (118, 119). These observations in the human confirm the two-cell, two-gonadotropin concept for adequate E_2 synthesis but also
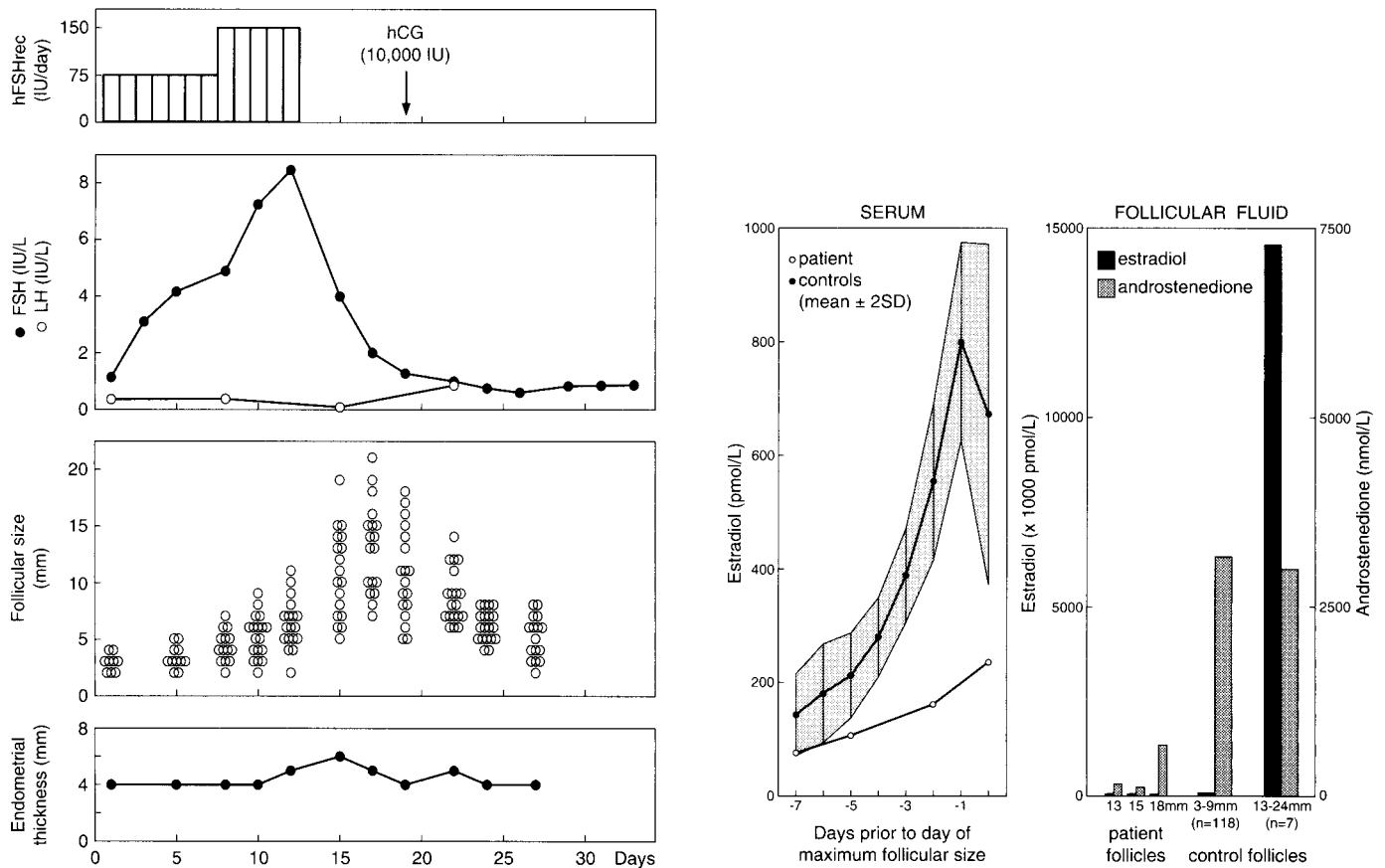
FIG. 3. Endocrine and sonographic observations in a single patient with isolated gonadotropin deficiency receiving daily intramuscular injections of human recombinant FSH (hrFSH). Serum FSH and LH levels, follicle diameter, and endometrial thickness (as assessed by TVS) are indicated in the *left panel*. Serum estradiol levels and follicle fluid estradiol and androstenedione concentrations (three follicles, 13–18 mm in diameter) from the patient and from regularly cycling controls [both nondominant (3–9 mm) and dominant (13–24 mm) follicles] are depicted in the *right panel*. HCG, Human chorionic gonadotropin. [Reproduced with permission from B. C. Schoot *et al.*: J Clin Endocrinol Metab 74:1471–1473, 1992 (87). © The Endocrine Society.]

demonstrate convincingly that increased E_2 production is not mandatory for normal follicle growth up to the preovulatory stage. These observations are fully supported by more recent similar studies in the monkey. Follicular growth and oocyte maturation in LH-deficient macaques did occur with FSH alone (120), and fertilization rates of oocyte obtained from recFSH/GnRH antagonist-treated monkeys were not compromised (121).

It is still uncertain whether estrogen receptors are present on granulosa cells from higher primates, including the human. Stouffer and colleagues were unable to show estrogen receptors on primate granulosa cells by immunocytochemistry (122), whereas androgen receptors could be demonstrated on both theca and granulosa cells (123). However, this technique may not be sensitive enough to detect low levels of receptors, and some investigators have recently described low levels of estrogen receptor mRNA as well as the protein in human granulosa cells from preovulatory follicles (124, 125). Moreover, direct effects have been described of the antiestrogen clomiphene citrate on E_2 synthesis by cultured human granulosa cells (126). As opposed to rats, diethylstilbestrol administration in juvenile primates resulted in decreased numbers of medium sized developing antral follicles (127). In addition, studies in hamsters have suggested that estrogens exert inhibitory effects on follicle function (128).

Collectively, these data suggest that in the human, E_2 is not required for follicle development. It appears that, under normal conditions, augmented E_2 synthesis is merely associated with dominant follicle development, where growth of the follicle is, in fact, driven by other nonsteroidal (growth) factors (see also *Section II.F*). This concept may also bear significance for our thinking regarding underlying causes of anovulation, in particular in polycystic ovaries. Follicles may cease to mature due to defective intraovarian regulatory mechanisms rather than the absence of aromatase enzyme induction *per se* (129).

During the follicular phase of the normal menstrual cycle E_2 is clearly important for other crucial physiological processes such as stimulation of endometrial proliferation, cervical mucus production, and induction of the midcycle LH surge and subsequent ovulation. Whether oocyte maturation in the human requires exposure to estrogens remains unclear at this stage (130–132).

D. In vivo regulation of follicle maturation in the monkey

A series of *in vivo* studies in the monkey has systematically addressed endocrine factors regulating follicle growth (for comprehensive reviews see Refs. 133–137). A significant proportion of these experiments have subsequently been confirmed in the human (see *Section II.E*). Surgical ablation of the dominant follicle in the late follicular phase of the cycle blocked the midcycle gonadotropin surge and ovulation. These observations indicate that no other follicles from the recruited cohort were capable of replacing the dominant follicle, presumably due to atretic changes. New follicle recruitment occurred in response to a rise in endogenous FSH levels, similar to ovarian response after removal of the corpus luteum. The duration until the next ovulation was 12 days, which equals the normal follicular phase of the cycle. There-

fore ovulation was delayed after follicle cautery and advanced after luteectomy (138). Ovarian response to exogenous gonadotropins (as estimated by rising serum E_2 levels) was equal, regardless of whether gonadotropins were administered in the follicular or midluteal phase of the cycle (139). By repeated cautery of the dominant follicle, it was also shown that the midcycle gonadotropin surge of the preceding cycle plays no role in follicle recruitment for the subsequent cycle (140).

Dominant follicle selection, and subsequent asymmetrical ovarian estrogen output, occurs around the midfollicular phase (141, 142). The dominant follicle requires continued though reduced support by FSH. In fact, growth of a single dominant follicle could be sustained in GnRH antagonist-treated monkeys by the administration of exogenous FSH in decremental doses (Fig. 4) (143), suggesting enhanced sensitivity for FSH when the dominant follicle matures (see also *Sections II.B* and *II.E2*). The dominant follicle continued its development despite relatively low late follicular phase FSH concentrations, incapable of stimulating growth of less mature follicles. Subsequent experiments in the monkey model have addressed the significance of FSH for single dominant follicle selection. Early follicular phase administration of E_2 caused a significant reduction in serum FSH and a lengthening of the follicular phase (144). Moreover, administration of antiestrogen antibodies in the early to midfollicular phase gives rise to elevated serum FSH levels, which interferes with single dominant follicle selection resulting in ongoing maturation of additional cohort follicles (145, 146).

The above mentioned experiments show similar responsiveness of the ovary to endocrine changes in either the luteal (147) or the follicular phase and provide *in vivo* evidence for the concept that gonadotropin-responsive follicles are maintained throughout the entire cycle. Follicles can be stimulated to ongoing and gonadotropin-dependent development when the appropriate endocrine signal (*i.e.* elevated serum FSH levels) is operative. Under normal conditions, elevated FSH concentrations are present during the luteo-follicular transition only. Augmented E_2 production by the most mature (dominant) follicle starting around the midfollicular phase causes a subsequent decrease in FSH levels due to negative feedback effects of E_2 on the hypothalamic-pituitary axis. The dominant follicle restricts ongoing maturation of other, less mature follicles from the cohort since FSH levels drop below their threshold for stimulation of gonadotropin-dependent growth. The dominant follicle is spared from the inhibitory influence of reduced FSH stimulation because of increased sensitivity to FSH (see also *Sections II.B* and *II.E.2*).

E. The FSH threshold and window concept for in vivo follicle growth

1. FSH threshold and follicle recruitment. Due to the demise of the corpus luteum and the subsequent decrease in estrogen production (148), FSH levels rise at the end of the luteal phase of the human menstrual cycle (149). This intercycle rise is closely synchronized with ovulation, and FSH levels start to increase 12 days after the preceding LH surge (150). As mentioned previously, initiation of growth of primordial follicles occurs continuously and in a random fashion. Fol-
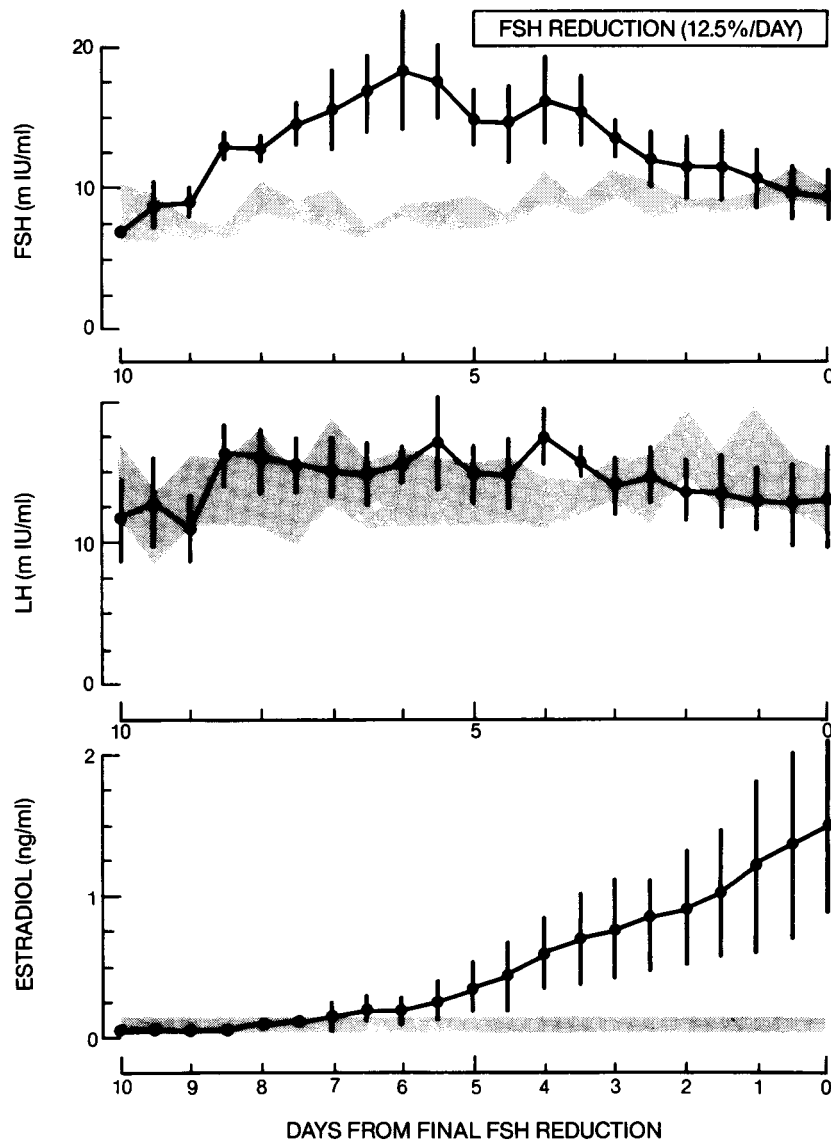
FIG. 4. Serum hormone levels (mean ± SEM; *solid line*) in four GnRH antagonist-treated monkeys during pulsatile infusion of LH and FSH. FSH infusion was reduced by 12.5%/day after a rise in serum estradiol levels, whereas LH input was kept constant. The *shaded areas* represent control values (n = 4). Note that estradiol levels continue to increase despite decreasing FSH serum levels. [Reproduced with permission from A. J. Zeleznik and C. J. Kubik: *Endocrinology* 119:2025–2032, 1986 (143). © The Endocrine Society.]

licle growth will eventually cease and follicles will enter atresia if the appropriate endocrine signal is lacking. Although each follicle may have an equal potential to reach full maturation, only follicles that happen to be at a more advanced stage of development during the intercycle rise in FSH will gain gonadotropin dependence. The concept that FSH concentrations above a certain level, referred to as the 'FSH threshold,' are needed for ovarian stimulation was first introduced by Brown in 1978 (151) and substantiated more recently by Schoemaker and colleagues. The individual variation in FSH serum levels at which follicle growth was initiated could be assessed to be between 5.7 and 12.0 IU/liter with the use of intravenous administration of gonadotropins in PCOS patients (152, 153). Moreover, multifollicular growth was shown to be associated with higher FSH concentrations above the threshold (153) (Fig. 5), using a low-

dose incremental protocol for FSH induction of ovulation. Each growing follicle has a threshold requirement for stimulation by circulating FSH. The threshold level should be surpassed to ensure ongoing preovulatory follicle development. This process of rescue of a cohort of follicles from atresia by FSH stimulation is referred to by most authors as 'recruitment.' The recruited cohort represents a group of follicles at a comparable (but not identical) developmental stage. This group of follicles, by chance, happened to leave the pool of resting follicles around the same period of time several months before. In contrast, other investigators reserve this term for the initiation of growth of primordial follicles (12) (see also *Section II.A*).

Morphological and endocrine studies suggest that healthy early antral follicles less than 4 mm in diameter are present throughout the cycle (89), in keeping with the concept that
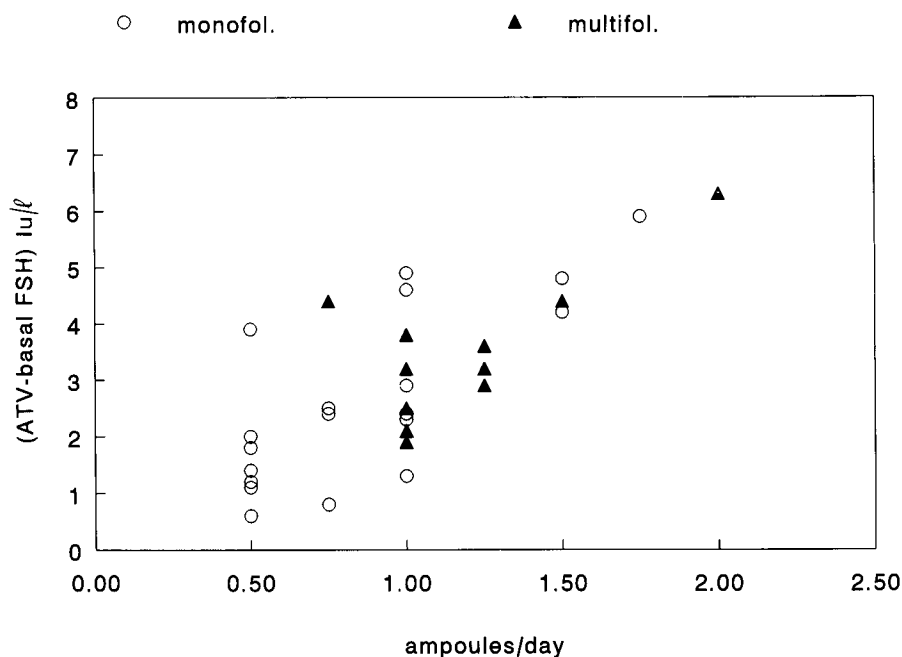
FIG. 5. Increase in FSH serum levels from basal to above the assessed threshold (ATV, above threshold dose) related to the number of FSH ampoules infused per day in 16 PCOS patients treated with a low-dose step-up regimen for gonadotropin induction of ovulation. It was proposed that multifollicular development (▲) is associated with higher FSH levels above the threshold and more ampoules per day as compared with patients presenting with monofollicle development (○). [Reproduced with permission from M. van der Meer *et al.*: *Hum Reprod* 9:1612–1617, 1994 (153).]

follicles are continuously available for stimulation by FSH. At the end of the luteal phase, the largest healthy follicles observed by morphological criteria have been described to be between 2 to 5 mm in diameter (10, 89, 154), and the number of recruitable follicles present is believed to be between 10 and 20 for both ovaries. Granulosa cells obtained from follicles in the late luteal phase are significantly more sensitive to FSH stimulation (as assessed by FSH-induced estrogen production *in vitro*) (154), suggesting that these healthy follicles will be recruited for the next cycle. The largest healthy follicles at the start of the follicular phase of the cycle have been reported to exhibit a diameter between 4 and 8 mm (94, 155), and no morphological differences exist between these follicles. These observations strongly suggest that the dominant follicle is selected at a later stage of the follicular phase of the cycle. Indeed, exogenous HMG administered during different phases of the menstrual cycle is most effective in stimulating follicle recruitment if administered during the late luteal or early follicular phase (156).

Elegant experiments in the human have further substantiated the FSH threshold concept and have generated additional support for the notion that follicles ready to be recruited are present throughout the menstrual cycle. Removal of the dominant follicle in the late follicular phase, or luteectomy in the luteal phase during gynecological surgery, results in new follicle recruitment and subsequent ovulation (157–159). Enucleation of the corpus luteum in 10 women was followed by an immediate and rapid decline of E_2 and progesterone levels. This was followed by rising FSH levels, renewed follicle growth, and ovulation within 16–19 days after enucleation (159). These experimental results are in full

agreement with observations in the monkey model after similar intervention and indicate indeed that suppressed gonadotropin secretion (due to corpus luteum or dominant follicle steroid production) is responsible for inhibition of more advanced follicle maturation. Moreover, these observations are in keeping with the notion that final and gonadotropin-dependent follicle growth preceding ovulation takes approximately 14 days, coinciding with the follicular phase length of the menstrual cycle. If the intercycle rise in serum FSH is shortened by the early to midfollicular phase administration of GnRH antagonist, follicle growth is arrested and new follicle recruitment will follow once medication is withdrawn (160, 161).

2. FSH window and single dominant follicle selection. In follicles less than 10 mm, the aromatase enzyme is poorly expressed (62) and intrafollicular E_2 levels are low (Fig. 3) (57, 75, 162). This also holds true for follicles in the early follicular phase of the menstrual cycle. E_2 production, however, can be stimulated rapidly *in vitro* by adding FSH to the culture medium (59, 68, 92). It cannot be readily explained why E_2 levels remain low despite maximum FSH stimulation in the early follicular phase (163). Intraovarian modification of FSH action may be involved (see *Section II.F.3*). Under normal conditions, the fate of developing antral follicles is closely associated with their ability to create an estrogen-rich intrafollicular environment, as discussed previously. It may be proposed that the follicle selected to gain dominance is the one that has most rapidly acquired the highest sensitivity for FSH. This may be the follicle that was at the most advanced developmental stage when recruited. Indeed, FSH respon-

siveness of cultured granulosa cells (obtained from follicles at various stages of development) has been shown to be dependent on follicle size, with more pronounced E_2 production by cells obtained from larger follicles (59, 68, 92, 162). Responsiveness to FSH stimulation is also increased in preovulatory follicles (164). In addition, in the late follicular phase, steroidogenic function of granulosa cells from the dominant follicle is also stimulated by LH (165). Finally, observations in the monkey suggest that increased vascularization of individual follicles (resulting in the preferential exposure to circulating factors) may also be instrumental in the selective maturation of preovulatory follicles (166).

Consequently, the FSH threshold for a given follicle is not fixed but is dependent on its developmental stage and therefore changes over time. Indeed, experiments applying GnRH antagonist for 3 consecutive days in the mid- or late follicular phase of the cycle have shown convincingly that the developing follicle becomes more resistant to gonadotropin withdrawal as it becomes more mature (160). Midfollicular administration of GnRH antagonist may induce a transient follicular arrest without triggering new folliculogenesis (167) or complete follicle maturation arrest and new follicle recruitment (161), depending on the magnitude and duration of gonadotropin suppression.

FSH serum levels steadily decrease during the mid- to late follicular phase of the menstrual cycle. The follicle that has gained dominance is less dependent on continued support by high early follicular phase FSH levels. However, circulating FSH levels are suppressed to a concentration below the threshold for remaining follicles from the recruited cohort. These follicles will therefore cease to mature and undergo atresia. Hence, development of the most mature follicle, closely associated with increased E_2 production, secures selection of a single dominant follicle. The FSH 'gate' (168) or 'window' (169, 170) (Fig. 6, *upper panel*) concept has been introduced to emphasize the significance of a transient elevation of FSH above the threshold. This concept emphasizes the importance of time (*i.e.* duration of elevated FSH levels) rather than dose (magnitude of FSH elevation) for single dominant follicle selection. Previous studies by our own group in 16 female volunteers have characterized follicular phase patterns of FSH serum levels and investigated correlations between decremental FSH levels and dominant follicle development (163) (see also Table 1 and Fig. 7, where the number of volunteers has been extended to 42). This decrease may be due to negative estrogen feedback on the hypothalamic-pituitary axis (168). However, it seems that the initiation of declining serum FSH levels precedes augmented ovarian estrogen output. We have observed a clear association between the magnitude of decrease in endogenous FSH serum levels and the E_2 rise, indicating that the duration of FSH stimulation (duration of serum FSH above the threshold) is a major determinant for ovarian E_2 production (163).

Indeed, a more pronounced but transient elevation of serum FSH concentrations above the threshold in the early follicular phase of the normal menstrual cycle (administration of 450 IU FSH on cycle day 2) did not result in multiple follicle development and enhanced E_2 production during the late follicular phase. In sharp contrast, low doses of FSH administered during the mid- to late follicular phase (starting
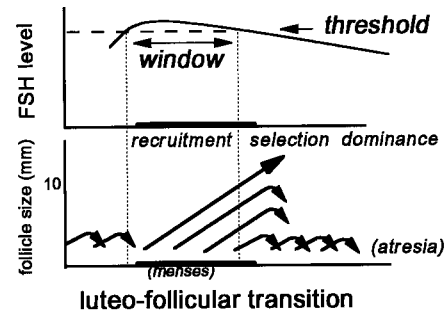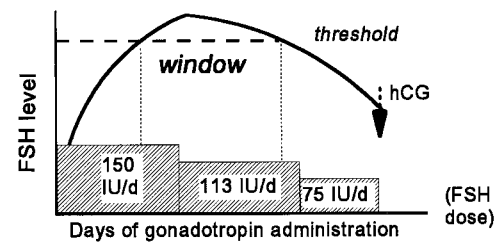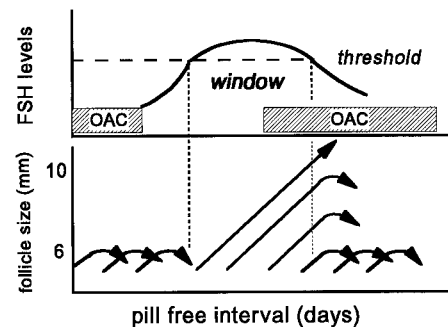
FIG. 6. Schematic representation of the intercycle rise in serum FSH levels (FSH threshold/window concept), and follicle growth dynamics (recruitment, selection, and dominance) during the follicular phase of the normal menstrual cycle (*upper panel*). FSH serum levels during the follicular phase of gonadotropin induction of ovulation using a step-down dose regimen (starting dose of 150 IU/day) are depicted in the *middle panel*. FSH serum levels and follicle growth during the 7-day pill-free interval following combined steroid contraceptive pills (OAC) are indicated in the *lower panel*. The FSH threshold is the serum level required for stimulation of ovarian activity. The FSH window represents the number of days when FSH concentrations remain above the threshold. Recruitment represents the transition from gonadotropin-independent to gonadotropin-dependent follicle development (follicles are rescued from their destiny to undergo atresia by the intercycle rise in FSH). Selection refers to the process where a single follicle gains dominance over the remaining follicles from the recruited cohort.

TABLE 1. Endocrine and sonographic characteristics of the follicular phase of the normal menstrual cycle in 42 volunteers[#]

	Median (range)
Cycle day 1	
FSH concentration (IU/liter)	4.0 (0.9–9.2)
E_2 concentration (pg/ml)	41 (20–129)
Follicle diameter (mm)	6 (4–15)
FSH	
Maximum	
Concentration (IU/liter)	6.6 (4.3–12.5)
Cycle day	5 (1–9)
Minimum	
Concentration (IU/liter)	3.3 (0.8–5.7)
Cycle day	13 (8–16)
Decrease	
IU/liter/day	0.4 (0.2–0.8)
Dominance[a]	
Follicle size (mm)	10 (6–15)
Cycle day	7 (3–15)
E_2 concentration (pg/ml)	52 (25–112)
Preovulatory (day LH surge)	
Follicle size (mm)	21 (15–26)
E_2 concentration (pg/ml)	236 (101–415)
Cycle day (LH surge)	16 (11–25)

[#] Blood sampling was performed daily. Transvaginal ultrasound was performed every 1–2 days. Median and range for cycle length was 28 (25–35) days. [Derived from Refs. 163, 206, and Schipper *et al.* (unpublished observations).]

[a] Dominance is defined (published previously; Refs. 163, 206) as; 1) follicle largest in size as assessed by transvaginal ultrasound, 2) at least 2 mm larger as compared to remaining follicles, and 3) optical fit in growth curve of dominant follicles.

on cycle day 4) did elicit a significant rise in serum E_2 levels (Fig. 8) (171) (I. Schipper and B. C. J. M. Fauser, unpublished observations). Moreover, selection of a single dominant follicle is also prevented if high FSH levels are sustained in hyperstimulation protocols for IVF. The magnitude of multiple follicle growth in IVF patients has been shown to be proportional to the late follicular phase accumulation of FSH in serum (172). These experiments confirm that the duration (related to the window concept) rather than the magnitude (threshold concept) of FSH stimulation determines the number of developing follicles. We have recognized the crucial role of decremental serum FSH levels for single dominant follicle selection under normal conditions and have attempted to develop a decremental ('step-down') dose regimen for gonadotropin induction of ovulation for treatment of anovulatory infertility (see *Section III.D*) (Fig. 6, *middle panel*). It could be demonstrated, indeed, that growth of the dominant follicle is sustained despite reduced late follicular phase stimulation by decremental doses of exogenous FSH (173).

On the basis of previous studies it has been proposed that inhibin is an unlikely factor to play a significant role in dominant follicle feedback actions, since it appears that several antral follicles contribute equally to ovarian immunoreactive inhibin secretion. Moreover, inhibin serum levels did not differ in blood draining the ovary bearing the dominant follicle compared with blood from the contralateral ovary (174). Inhibin levels did not change during the early follicular phase. However, early inhibin immunoassays suffered from extensive cross-reactivity with potentially inactive precursors. Exciting new information has become avail-
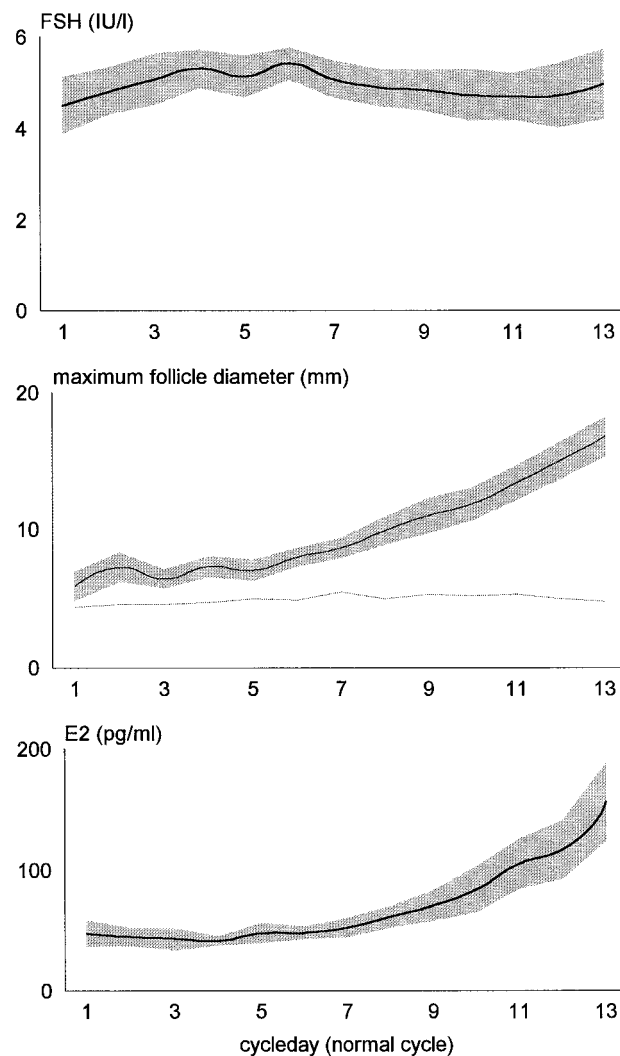
FIG. 7. Follicular phase serum FSH levels (*upper panel*), maximum follicle diameter (mm) (*middle panel*), and E_2 levels (*bottom panel*) (mean and 95% confidence intervals) according to cycle day in 42 young volunteers with normal ovarian function. The *dotted line in the middle panel* indicates mean size of all observed follicles.

able recently since the development of new sandwich assays using monoclonal antibodies directed against the βB-subunit (the α-subunit combined with βB constitutes inhibin B) or against βA (inhibin A). Follicular phase serum patterns of inhibin A appear to be comparable to previously used less specific assays (175). In contrast, a profound rise in inhibin B serum levels was observed early in the follicular phase, suggesting that it is secreted by recently recruited cohort follicles in response to FSH. This rapid rise in inhibin B occurs just after the intercycle rise in FSH. It may be proposed that inhibin B limits the duration of the FSH rise (narrowing the FSH window) through negative feedback at the pituitary level and may therefore be crucial for mono follicle development. Elevated early follicular phase FSH levels in elderly ovulatory women were shown to be associated with decreased inhibin B secretion, which may be due to a reduced number of recruitable follicles in women of advanced reproductive age (176).

FIG. 8. Daily follicular phase FSH and E_2 serum levels (mean and SE) in 13 control cycles (○-○), after a single intramuscular injection of 600 IU FSH on cycle day 2 (●-●), 600 IU on day 2 plus 75 IU FSH daily from cycle day 4 onward (□-□), or 600 IU on day 2 plus 150 IU daily from cycle day 4 onward (■-■). Thirteen regularly cycling women were studied during four cycles. This study suggests that in the early follicular phase a distinct but short elevation of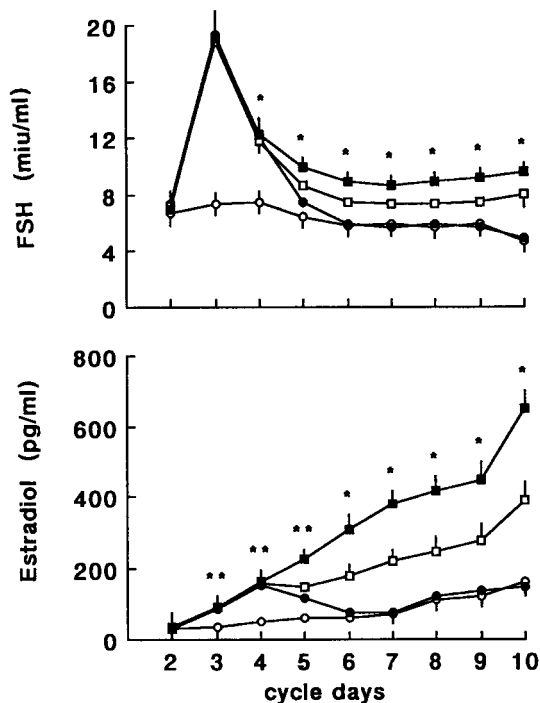 serum FSH levels above the threshold does not result in increased ovarian response. In contrast, ovarian activity is significantly enhanced due to a modest but extended mid- to late follicular phase increase in FSH (effectively widening the FSH window). [From Lolis DE, Tsolas O, Messinis IE. The follicle-stimulating hormone threshold level for follicle maturation in superovulated cycles. *Fertil Steril* 1995; 63:1272–1277; Reproduced with permission of the publisher, The American Society for Reproductive Medicine (formerly The American Fertility Society).]

3. Dominant follicle development. The ability to monitor growth of a large antral follicle, by means of transabdominal pelvic ultrasound, was originally described in an anovulatory patient during gonadotropin induction of ovulation (177). This noninvasive technique has allowed large-scale characterization of dominant follicle growth during the normal menstrual cycle (178–200). Follicles could be visualized from 8–10 mm onward (181), and usually two to three follicles per ovary could be identified (188, 189). Follicle size assessed by ultrasound has been compared with follicle volume as determined by the amount of fluid collected after puncture (182), or with follicle size during laparoscopy (190). Interobserver variability has been shown to be limited (200). Timing of ovulation could be predicted using ultrasound (180, 186, 187, 193), and the mean size of the preovulatory follicle reported in various studies ranged between 20 and 27 mm (201). On an individual basis, a high correlation was observed between follicle size and E_2 serum levels (179, 185). However, major individual variability in size of preovulatory follicles (191) results in linear regression, but low overall correlations between follicle size and serum E_2 levels (75, 163,

185, 188, 195, 199). Growth of the dominant follicle is generally mentioned to be linear, with a mean daily growth rate around 2–3 mm (163, 183).

Since 1985 the transvaginal route has been introduced for pelvic ultrasound (202, 203), allowing enhanced imaging resolution and a more reliable assessment of changes in number and size of small follicles (197). Growth of dominant and nondominant follicles has been studied extensively by our group using transvaginal sonography (TVS) (for review see Refs. 204 and 205). Up to 11 follicles (>2 mm in diameter) could be observed throughout the cycle in each ovary, and a dominant follicle could be visualized from 10 mm onward (Fig. 9) on cycle day 9 (Table 1). The size of nondominant follicles visualized by TVS always remains below 11 mm (163, 206). The ultrasound observation of dominant follicle selection correlates strongly with a sudden increase in serum E_2 concentrations ($r = 0.84$; $P < 0.001$), indicating that visualization of the dominant follicle coincides with enhanced E_2 synthesis (163), as has been shown previously by augmented E_2 levels in venous blood draining the ovary bearing the dominant follicle (88). This *in vivo* ultrasound observation also agrees fully with and extends previous studies (as discussed in *Section II.B*) showing that: 1) Aromatase activity *in vitro* is only observed if granulosa cells were obtained from follicles beyond 8 mm in size. 2) Augmented intrafollicular E_2 concentrations (and positive immunostaining of the P-450AROM enzyme) only in follicles beyond 10 mm. It could also be demonstrated that early follicular phase FSH levels decrease before the onset of a rise in serum E_2 concentrations (163, 204), which supports the notion that other ovarian factors (like for instance inhibin B) are to be held responsible for narrowing the FSH window.

F. Modulation of FSH action

In the previous section we have focused on the significance of patterns of serum FSH concentrations for follicle recruitment and selection. However, in addition to the quantity of hormone released by the pituitary, the FSH signal may also be altered by a difference in the distribution of various FSH isoforms, as well as by interference with FSH binding to the receptor, or by interference with postreceptor signal transduction by, for instance, growth factors. It may also be hypothesized that signal transduction after ligand binding may be influenced by the existence of various forms of transmembrane FSH receptors (so-called 'splice variants') (for review see Ref. 207).

1. Heterogeneity of FSH. Variant forms of FSH are synthesized and secreted by the anterior pituitary, on the basis of differences in oligosaccharide structure of these glycoproteins as well as the number of incorporated terminal sialic acid residues. FSH heterogeneity should be considered as a continuum of molecular forms, each with distinct physiochemical characteristics. Glycoprotein isohormones with different carbohydrate side chains can be separated by their differences in charge. Depending on the sophistication of techniques used, up to 20 isoforms have been characterized for human FSH. Heavily sialylated (more acidic) FSH has been described to exhibit reduced receptor binding and *in vitro*
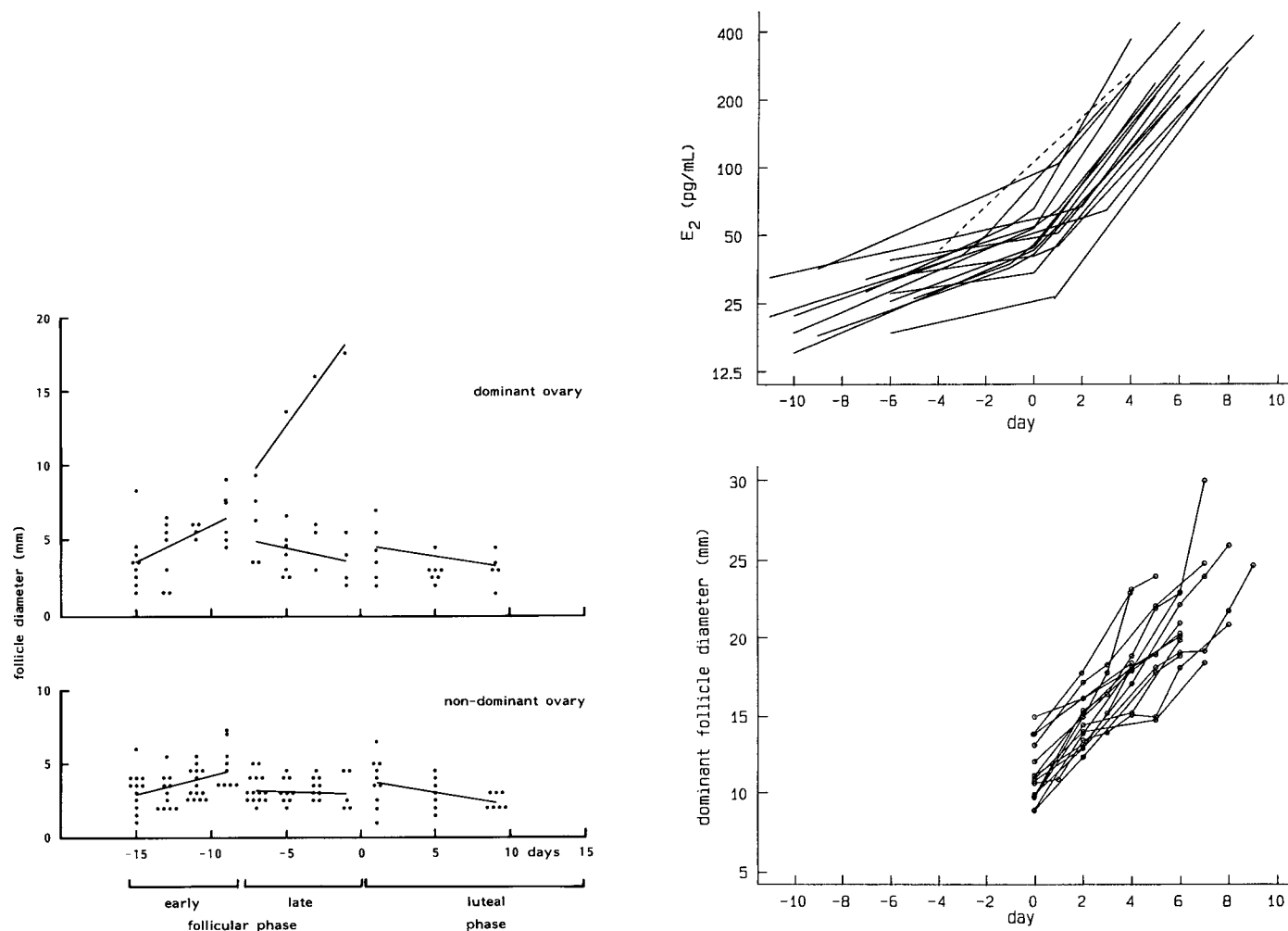
FIG. 9. Diameters of individual ovarian follicles both in the dominant and contralateral ovary in a single regularly cycling woman. Day 0 represents the day of the LH surge (*left panel*). Rise in serum estradiol levels and growth of the dominant follicle were synchronized around the first day of visualization of the dominant follicle by TVS in 16 regularly cycling female volunteers (*right panel*). [From Pache TD, Wladimiroff JW, DeJong FH, Hop WC, Fauser BC. Growth patterns of nondominant ovarian follicles during the normal menstrual cycle. *Fertil Steril* 1990; 54:638–642; and van Santbrink EJP, van Dessell TJHM, Hop WC, DeJong FH, Fauser BCJM. Decremental follicle stimulating hormone and dominant follicle development during the normal menstrual cycle. *Fertil Steril* 1995; 64:37–43. Reproduced with permission of the publisher, The American Society for Reproductive Medicine (formerly the American Fertility Society).]

bioactivity, whereas circulating half-life of these forms is extended. These forms may be desialylated in the circulation. In contrast, basic isoforms have been described to be more biopotent *in vitro* (2- to 5-fold), whereas the circulating half-life is significantly reduced (for comprehensive reviews see Refs. 208 and 209).

Effects of estrogens on the *in vivo* isohormone profile of FSH have been repeatedly established. In fact, changes were found during the normal menstrual cycle, as well as after menopause. In a small number of women, more basic isoforms were described to be present at midcycle (210–212). Estimates of changes in FSH heterogeneity, as assessed by *in vitro* bioassays, during the menstrual cycle are contradictory (210, 213, 214) and appear to be dependent on the assay system used. It has been speculated that ovarian follicles are recruited in the early follicular phase (when gonadal steroid feedback is low) predominantly by more acidic FSH isoforms, whereas follicle selection and rupture later during the

follicular phase is dependent chiefly on more basic FSH isoforms. However, the net effect of a predominance of more bioactive but shorted half-life forms on the overall *in vivo* biopotency is unknown at this stage, and therefore the physiological significance of described changes in FSH isoforms remains open for speculation.

2. Direct interference with FSH action. It has been proposed that low molecular weight proteins specifically interfering with FSH receptor binding are present in serum (215). In addition, a high molecular weight FSH receptor binding inhibitor was partially purified from human follicle fluid by the same group of investigators (216). However, these proteins have never been fully characterized, and the physiological relevance remains uncertain (for review see Ref. 207). Cell lines transfected with the human FSH receptor may prove a valuable tool with which to study further the pathophysiological relevance of inhibition of FSH receptor activation (217–219a).

3. Intraovarian interference with FSH action by growth factors.
Serum FSH concentrations are maximal in the early follicular phase of the menstrual cycle. In contrast, circulating E_2 levels start to rise around the midfollicular phase coinciding with the visualization of a dominant follicle by ultrasound. E_2 production, however, can be stimulated rapidly *in vitro* by adding FSH to the culture medium (59, 68, 92), and it cannot be readily explained why early follicular phase E_2 levels remain low despite maximum FSH stimulation (163). The lag period between maximum FSH stimulation and augmented ovarian E_2 output may be explained by intraovarian inhibition of FSH action early in the follicular phase or enhancement of FSH action within the dominant follicle (Fig. 10). The dominant follicle continues to mature despite decreased stimulation by lower late follicular phase FSH concentrations. This observation of decreased dependence of the dominant follicle on FSH stimulation (as discussed extensively in *Section II.E*) strongly suggests that the FSH signal is modified within the ovary, either at the level of FSH binding to the receptor or by interference with postreceptor signal transduction. In addition, the intrafollicular rise in E_2 levels of the dominant follicle was believed to be responsible for the decreased need for stimulation by FSH through autocrine short loop up-regulation (220). However, it is now clear that follicles can mature fully without a concomitant rise in E_2. This observation strongly suggests that other (intraovarian) factors in fact drive growth of the follicle, and disturbed intraovarian regulation may prove to be crucially important for cessation of follicle development in PCOS patients. Moreover, a 2.5-fold difference in maximum early follicular phase FSH serum concentrations — not correlated with any other follicular phase parameter, such as length or follicle growth characteristics (163) — observed in a group of young women presenting with normal ovarian function suggest distinct differences in the individual FSH threshold. This observation implies differences in intraovarian regulation under normal conditions.

After initial studies regarding effects of different growth factors on FSH-stimulated granulosa cell function *in vitro* (for review see Ref. 221), numerous studies have been undertaken regarding the potential physiological significance of growth factors for intraovarian modification of FSH action (12, 164, 222–225). The majority of growth factors, such as insulin-like growth factors (IGF) (226), transforming growth factor-β, fibroblast growth factor, and activin (227), have been shown to enhance FSH action *in vitro*. In contrast, other growth factors have been shown to inhibit FSH-stimulated E_2 biosynthesis by cultured human or primate granulosa cells, including inhibin (228), epidermal growth factor (229–231), and IGF binding protein (IGFBPs) (232). Decreased follicle fluid epidermal growth factor and transforming growth factor-α concentrations have been described when follicles mature (233–235). Moreover, white blood cell-derived cytokines, such as like tumor necrosis factor, interferon, or interleukins, have been proposed to be relevant for human ovarian physiology (236).

Certainly, overwhelming evidence is available regarding major changes in the IGF system during follicle development in the human ovary (237). Expression of IGF-II and their binding protein (IGFBPs), as well as IGF receptors, has been shown to be dependent on the developmental stage of the follicle (238, 239). IGFBP-3 was shown to exhibit structural similarity with the FSH-binding inhibitor (240), and the IGFBP profile in follicle fluid has been described to vary during follicle development, independent from changes in serum (241). Moreover, proteases capable of specifically decreasing the level of IGFBP-4 could be demonstrated in estrogen-dominant follicle fluid only (242), suggesting that more bioavailable IGF-II is available to synergize with gonadotropins in the dominant follicle. It should be noted, however, that growth of follicles could be induced by exogenous FSH in a patient with Laron-type dwarphism (low endogenous IGF-I secretion due to a familial GH receptor defect) (243), suggesting that IGF-I is not required for normal ovarian function. Conclusive *in vivo* evidence that any of the above mentioned growth factors play a distinct role in human ovarian physiology is lacking as yet.
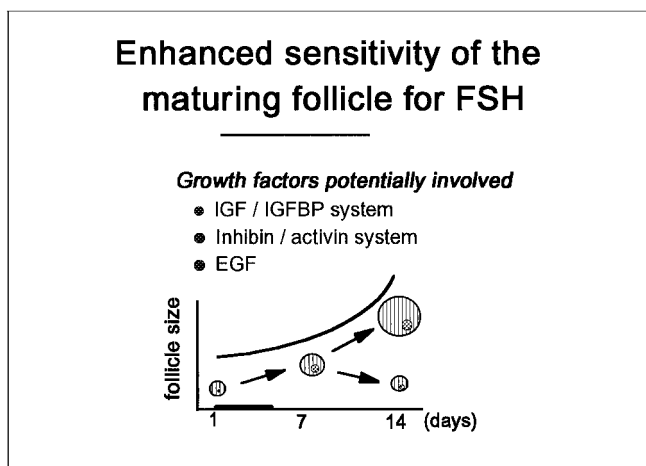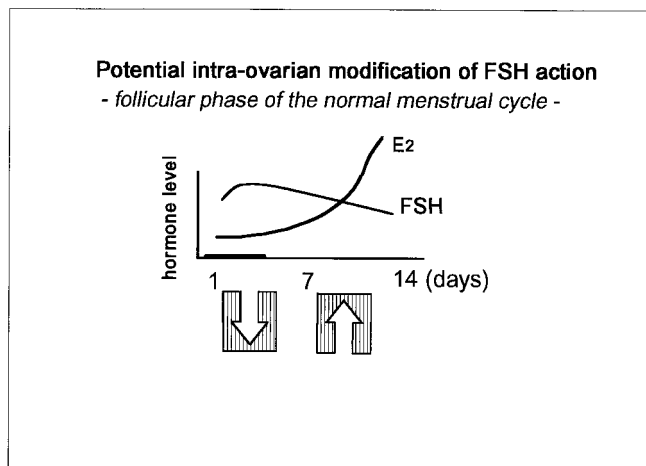

FIG. 10. Schematic representation of potential modification of FSH action within the ovary, being either inhibitory in the early follicular phase, or stimulatory in the late follicular phase (*top panel*), and growth factors potentially involved in late follicular phase enhancement of FSH action (*bottom panel*).

III. Gonadotropin Induction of Ovulation

A. The concept of monofollicle growth in anovulatory patients

Exogenous gonadotropins have been widely used for the treatment of anovulatory infertile women since 1958 (for comprehensive reviews see Refs. 244–250). Although commercially available gonadotropin preparations are generated through extraction of human urine, the first described application involved gonadotropins obtained from human pituitaries (251). HMG preparations (FSH to LH activity ratio, 1:1), obtained from urine of postmenopausal women, are administered to stimulate follicle growth, whereas pregnant women provide the urine source for hCG preparations (with LH-like activity) to induce ovulation. During the first decade of clinical use, various dose regimens, such as fixed, intermittent, or flexible incremental or decremental doses, have been tested (252, 253). It should be realized that at that time ovarian response could only be estimated by indirect measures, such as palpation of ovarian size or assessment of cervical mucus production resulting from ovarian estrogen secretion. Tools to measure ovarian response after exogenous FSH have improved considerably over the years.

The great majority of anovulatory patients presently treated with gonadotropin preparations comprise normogonadotropic (*i.e.* normal serum FSH concentrations; World Health Organization, class II) anovulatory infertile women who failed to conceive during previous antiestrogen medication. The aim of this treatment modality is to approach normal conditions as closely as possible; *i.e.* maturation and ovulation of a single dominant follicle and subsequent singleton pregnancy. Characteristics of dominant follicle development during gonadotropin induction have been documented using ultrasound and serum estrogen assays (254–257). It should be stressed that the goal of induction of ovulation is completely different from 'controlled' ovarian hyperstimulation for IVF, where the goal is to interfere with selection of a single dominant follicle to obtain multiple oocytes for IVF. Therefore, the use of the term induction of ovulation for IVF is confusing and should be abandoned.

Although gonadotropin therapy has been shown to be fairly successful in terms of ovulation rates (reported in the literature between 60–100%) and cumulative pregnancy rates (reported between 20–75%), complication rates are high. Major complications include multiple pregnancies (258), ovarian hyperstimulation (259), and a high rate of early pregnancy wastage (260). The first two complications have been shown to be related to the magnitude of multiple follicle development as estimated by serum estrogen levels (261–263) and more recently by pelvic ultrasound (264). The high abortion rate has been suggested to be related to elevated LH levels (265, 266). In addition, a significant increase in the overall prevalence of multiple pregnancies over the last 10–20 yr has been established repeatedly in the literature (267–271), and gonadotropin induction of ovulation is certainly involved. Inherent problems include social difficulties, ethical considerations regarding fetal reduction (272, 273), perinatal morbidity, and increased health care costs (274).

A great individual variability in ovarian response to stimulation by FSH (so-called 'FSH threshold') was proposed in anovulatory patients (151). Moreover, Brown (151) stressed that only a small margin exists between an effective dose and a dose generating excessive ovarian response. Unfortunately, predictors for the FSH threshold of a given patient have not been identified. The concept of the FSH threshold in anovulatory patients was substantiated more recently (152, 153) with the use of intravenous administration of exogenous gonadotropins by pump. The threshold level was arbitrarily extrapolated from the first day a follicle beyond 12 mm could be observed by transabdominal ultrasound or TVS. No difference in the FSH threshold was observed, comparing HMG *vs.* FSH. Moreover, a 2-fold variation in individual threshold levels was observed, and higher FSH serum levels above the assessed threshold were found to be associated with multifollicular growth (Fig. 5). Major individual variability in response to stimulation by exogenous FSH underscores the need for careful and frequent monitoring of ovarian response by ultrasound and/or rapid serum E_2 assays (257) and adjustment of doses on an individual basis. In general, the focus is to approach the individual threshold level prudently, to prevent serum FSH concentrations to increase far above the threshold. Differences in the FSH threshold level result in considerable variability in the duration of gonadotropin administration in the event that low initial doses are administered. Unaltered late follicular phase FSH serum levels in gonadotropin-induced cycles differ greatly from the follicular phase of the normal menstrual cycle. This condition may elicit growth of other cohort follicles and, as a result, induce multiple follicle development.

During the interphase from one menstrual cycle to the other, serum FSH concentrations surpass the threshold for stimulation of ongoing and gonadotropin-dependent follicle development. Serum FSH levels decrease steadily during the follicular phase, securing the formation of a single dominant follicle. Only this follicle reaches the full mature state despite diminished stimulation by FSH, whereas growth of the remaining less mature follicles in the cohort ceases due to insufficient support by FSH. The significance of this pattern of FSH stimulation is stressed by various intervention studies, both in the human and in the monkey model, as discussed extensively in *Sections II.D* and *II.E*. The threshold concept for induction of ovulation focuses only on the magnitude of ovarian stimulation by FSH, but ignores the element of time. In contrast, the FSH window concept emphasizes the importance of FSH concentrations surpassing the threshold for a limited period of time only. Decremental dose regimens for exogenous FSH may be more effective in inducing preferential growth of the leading follicle (Fig. 6, *middle panel*). This approach may have implications for gonadotropin induction of ovulation, as discussed later in this section. In addition to the gonadotropin dose, many other factors may influence treatment outcome. These conditions will first be discussed.

B. Conditions affecting treatment outcome

1. Patient-related factors. Women diagnosed as hypogonadotropic hypogonadism, by definition, suffer from inadequate stimulation of ovarian function. FSH serum levels are below

the threshold, and growth of follicles is arrested at a stage where further development becomes dependent on stimulation by gonadotropins. If FSH levels rise above the threshold, due to exogenous administration of gonadotropin preparations, ovarian response should be normal. Success and complication rates of gonadotropin induction of ovulation in these patients is indeed favorable (275–281). However, the great majority of patients presently treated with gonadotropins present with clomiphene-resistant normogonadotropic anovulation. Serum FSH and E_2 levels in these patients are within normal limits. Obviously, normal limits for both FSH and E_2 depend heavily on the phase of the menstrual cycle. As mentioned previously, maximum early to mid follicular phase FSH levels are twice as high as late follicular phase concentrations (see also Table 1). Moreover, even in young regularly cycling women the FSH threshold varies considerably (at least 2-fold). This variability is poorly recognized in the classification of anovulation on the basis of serum FSH assays. For a given anovulatory woman, FSH levels 'within the normal range' may simply mean FSH levels below the threshold for ovarian stimulation. Hence, only the intercycle rise in FSH above the threshold may be lacking in these patients.

Normogonadotropic anovulatory women frequently suffer from PCOS. This heterogeneous group of patients is characterized by ovarian abnormalities (polycystic ovaries) combined with distinct endocrine features (elevated serum LH and/or androgen levels) (282). Various lines of evidence indicate that early follicle development is normal in these patients, whereas anovulation is caused by disturbed dominant follicle selection (74). This abnormal condition may be caused by disturbed intraovarian regulation of FSH action (129), and therefore response to exogenous FSH may be different from normal. Hence, the presence or absence of ovarian abnormalities in patients may influence treatment outcome after exogenously administered gonadotropins. This may explain major differences in the FSH threshold and duration of stimulation needed to induce preovulatory follicle development in these patients.

Presently, the wish to establish a family is expressed later in life. Therefore, the population of women seeking help for infertility is increasing in age. It has been documented that cumulative conception rates after gonadotropin induction of ovulation are distinctly different when women under the age of 35 are compared with older women (276, 280).

Obesity frequently coincides with PCOS, and differences in pharmacokinetic characteristics of gonadotropin preparations (283), as well as clinical outcome (284, 285) related to body weight, have been reported. Moreover, other concomitant endocrine disorders such as hyperprolactemia or adrenal hyperandrogenemia may also affect treatment outcome.

2. Hormone preparation-related factors. Preparations of urinary gonadotropin have been continuously improved since its commercial introduction. HMG preparations contain similar (1:1 ratio) FSH and LH activity, as required by regulatory agencies (286, 287). The most significant improvements of HMG preparations over the years involved the introduction of 1) purified urinary FSH (with only minute amounts of LH),

2) highly purified urinary FSH (obtained through an affinity extraction procedure, removing virtually all of the contaminating proteins) (288), and 3) human recombinant FSH preparations (113, 289–291). Other recombinant glycoprotein preparations — such as recombinant hCG (292), LH (293), long-acting FSH (FSH-CTP) (294), short-acting (deglycosylated) FSH (295), and single-chain gonadotropins (296) — will be available soon. This fascinating development certainly provides the clinical investigator with a whole new set of tools with which to manipulate ovarian function. Moreover, the clinician will have the unique and challenging opportunity to tailor compounds and corresponding circulating half-lives according to the treatment goal and the individual needs of the patient. Different host cells produce recombinant FSH with different isohormone profiles (297). Therefore *in vivo* biopotency of a given distribution of FSH isoforms may vary. However, it is uncertain at this stage whether this approach my result in improved treatment outcome.

Since elevated LH levels are believed to be involved in poor reproductive outcome, many studies have been undertaken to test whether the administration of urinary FSH, as compared with HMG, may improve treatment outcome in PCOS patients. However, all published comparative trials have failed to show such an effect (298–301). Considerable batch-to-batch differences have been observed for urinary gonadotropin preparations (302–304), and therefore the amount of bioactive FSH administered may vary from patient to patient, or from cycle to cycle within the same patient. Clinicians should be aware of the fact that a clear discrepancy may occur between the number of ampoules administered and the amount of circulating bioactive FSH actually stimulating the ovaries. Differences in the isohormone profile of various preparations — with a predominance of more acidic or basic forms — may be involved (208, 209). Unfortunately, few studies have been undertaken focusing on immunoactive and bioactive FSH levels and ovarian activity during gonadotropin induction of ovulation in a clinical setting.

It appears that the route of administration (being either intraperitoneal or subcutaneous) does not represent a major factor in determining clinical outcome, although solid comparative studies have not yet been published. Some pharmacokinetic differences have been observed comparing both routes of administration (305). Patient convenience is certainly served using the subcutaneous administration route.

3. Other factors involved. Probably the most important single factor determining success and complication rates for gonadotropin induction of ovulation is response monitoring of the ovary. Presently, this can be performed through frequent TVS scanning of number and size of developing follicles, and rapid serum E_2 assays. Aims of ovarian monitoring are to assess the effective FSH dose, duration of FSH medication, timing of hCG to induce ovulation, and finally prevention of multiple follicle development. Correlations between serum E_2 levels and number and size of follicles have been studied (194, 306), and it was shown that E_2 production is the net result of all developing follicles. This is in sharp contrast to normal follicle development where estrogens are produced by a single dominant follicle only. It should also be realized

that the previously established correlation between follicle development and E_2 levels during gonadotropin induction of ovulation may change in the event that recombinant FSH is used instead of urinary gonadotropin preparations (115). There is ongoing debate whether ultrasound alone may suffice or whether both monitoring techniques should be combined to secure maximum safety (194, 263, 307–309). Various reports have emphasized the possibility of predicting chances for multiple pregnancies or ovarian hyperstimulation by ultrasound alone (264). However, high E_2 levels are associated with increased chances for ovarian hyperstimulation regardless of ultrasound findings (173, 306).

Concomitant medication, in addition to gonadotropins, may include: 1) dexamethasone suppression of adrenal androgen production (310); 2) GnRH agonists to suppress endogenous release of LH (and FSH) (311, 312); 3) dopamine agonists therapy in case of hyperprolactinemia; 4) GH in an attempt to improve ovarian responsiveness (313); and 5) luteal support either by hCG or progestins. The concept of the adjuvant administration of GH is appealing and represents an innovative attempt to transpose the concept of intraovarian regulation to clinical practice. Hsueh and colleagues showed in the rat model that GH augmented intraovarian IGF-I production (314) and granulosa cell differentiation (315). However, it was shown more recently that species differences exist, and that human granulosa cells exclusively produce IGF-II. Although amplification of gonadotropin action on the ovaries by GH could be repeatedly demonstrated in the human, these studies have failed to clearly establish an improvement of treatment outcome (316–318). All in all, none of the above mentioned options improved pregnancy rates in prospective randomized comparative trials.

The amount of hCG administered to induce ovulation may also vary. Ovarian hyperstimulation does not occur if hCG is withheld, and therefore various investigators have focused on triggering ovulation by other means such as GnRH or GnRH agonists in an attempt to reduce hyperstimulation rates (319). Moreover, recombinant LH with a shorter half-life will soon be available to trigger ovulation (293), which may also reduce chances of hyperstimulation.

There are several other factors that may affect treatment results but that are usually ignored when differences in outcome of various studies are compared. First line therapy of normogonadotropic anovulatory women usually involves anti-estrogen medication, and gonadotropin medication is only applied in case of 'clomiphene resistance.' This term, however, is poorly defined in the literature and a major discrepancy exists between doses applied and number of months treated. When cumulative pregnancy rates are reported, the duration of gonadotropin treatment (number of cycles included per patient) varies considerably (reported between 3 and 12 months) (for review see Ref. 249).

C. Commonly used step-up dose regimen

1. Conventional step-up protocol. Conventional step-up dose regimens for gonadotropin induction of ovulation are characterized by initial daily doses of two ampoules (= 150 IU of bioactive FSH). Doses may be increased after 5 days in the event that ovarian response is judged to be insufficient. This protocol has been the preferred dose regimen world wide since the early 1970s. Estimation of ovarian response changed over time from physical examination to (urine and later serum) estrogen assays, to abdominal ultrasound, and more recently TVS. Improved accuracy of response monitoring resulted in superior treatment outcome. For more detailed information regarding reported success and complication rates of this conventional high-dose regimen, see Table 2. Again, these data should be interpreted with great caution since patient diagnosis and age, response monitoring, and duration of therapy may vary from study to study. Collectively, these data suggest that this treatment modality is effective, with a relatively high complication rate (253, 275–279). This is now believed to be related to FSH serum levels being too far above the threshold in a great proportion of patients. However, few studies have focused on FSH serum levels and ovarian response during conventional step-up cycles (320–322).

2. Low dose, step-up protocol. Although originally developed on the East coast of North America (323), the low-dose

TABLE 2. Clinical outcome of conventional step-up regimens for gonadotropin induction of ovulation (starting dose 2 ampoules/day) in normogonadotropic anovulatory infertile women

	Author, published year, ref. no.					
	Thompson and Hansen, 1970 (253)	Oelsner *et al.*, 1978 (275)	Dor *et al.*, 1980 (276)	Healy *et al.*, 1980 (277)	Schwartz *et al.*, 1980 (278)	Wang and Gemzell, 1980 (279)
Patient diagnosis	PCO	WHO II	WHO II	WHO II	WHO II	PCO
No. of patients	212	318	348	40	88	41
No. of cycles	546	[a]	1137	159	249	77
Ovulatory cycles (%)	62	[a]	[a]	93	98	97
Conceptions						
per started cycle (%)	10	[a]	[a]	[a]	[a]	[a]
per ovulatory cycle (%)	[a]	[a]	[a]	[a]	20	[a]
Cumulative pregnancy rate (%)	26	21	50	75	45	66
Multiple pregnancy rate (%)	17	16	[a]	25	15	36
Abortion rate (%)	39	42	[a]	10	33	24
Hyperstimulation rate (%)[b]	1.1	4	[a]	14	1.4	12

PCO, Polycystic ovaries; WHO II, World Health Organization, class II (defined as normal serum gonadotropin and/or normal E_2 levels).
[a] No information provided.
[b] No uniform classification.

step-up regimen for gonadotropin induction of ovulation has been the preferred method of stimulation in Europe since 1990. This dose regimen is characterized by low initial daily gonadotropin doses ranging between one-half and one ampoule (38–75 IU of bioactive FSH), and doses are only increased by one-half ampoule per day after 14 days, in cases of insufficient ovarian response. See Table 3 for more detailed information regarding reported success and complications of this dose regimen. Overall, this treatment modality seems to be characterized by low complication rates, at the price of an extended duration of treatment and possibly a slightly diminished success rate (323–334).

This treatment modality is aiming at slowly and prudently reaching the FSH threshold for stimulation of ovarian activity, in an attempt to reduce the magnitude of serum FSH levels surpassing the individual threshold (335). As mentioned previously, the individual FSH threshold may vary considerably. This means that in a given patient, gonadotropins are administered for an extended period of time, and the amount is only augmented after 14 days of treatment in the event that a relatively high FSH threshold is operative. Pharmacokinetic studies have indicated that it takes approximately 5 days before steady state FSH levels are reached when similar gonadotropin doses are administered daily through the intraperitoneal route (283, 336). Therefore patients may be exposed to FSH levels that are too low to stimulate follicle growth for several weeks. It is uncertain whether ovaries may be sensitized by extended exposure to subthreshold FSH levels. Similar daily serum FSH levels were measured preceding hCG administration in patients treated with low-dose, step-up protocols (330) (E. J. P. van Santbrink and B. C. J. M. Fauser, unpublished observations). Hence, in the late follicular phase FSH serum levels remain above the threshold for an extended period of time, resulting in a wide FSH window even when low-dose step-up protocols are used. Improved treatment outcome, as compared with conventional step-up protocols, is likely due to the reduced magnitude of FSH levels surpassing the threshold when lower initial doses are used. Improved monitoring of ovarian response should not be ruled out as an additional important factor responsible for improved safety of treatment.

On the basis of preliminary findings it has been suggested that late follicular phase serum FSH levels diminish due to increased E_2 negative feedback *only* in a subset of patients exhibiting monofollicle development (153, 337). Observed differences in late follicular phase FSH concentrations comparing patients presenting with monofollicular *vs.* multifollicular development (337) suggest again that the magnitude of FSH accumulation, which seems unpredictable even during low-dose regimens, determines individual response. The conclusion seems justified that late follicular phase estrogen steroid feedback is overruled to a variable degree in patients treated with low-dose step-up gonadotropin doses.

D. Potential for a step-down dose regimen

Zeleznik and co-workers (145) studied the significance of decreasing FSH serum levels for single dominant follicle selection in the monkey model. Histological examination of ovaries 5 days after passive immunization of monkeys with estradiol antibody infusion revealed the presence of two or more large follicles, suggesting that interference with the FSH-suppressive actions of E_2 results in continued maturation of secondary follicles. Moreover, GnRH antagonist-treated monkeys were infused with human LH and FSH. The LH dose was kept constant and FSH doses were increased every 3–4 days until serum E_2 levels rose. Subsequent reduction of the FSH amount with 12.5% per day for 5 days was accompanied by continued follicle development and a further rise in serum E_2 levels (Fig. 4) (143). These *in vivo* observations indicate a reduced need of the dominant follicle for FSH, since this follicle continues to mature despite relatively low FSH levels, incapable of stimulating growth of less mature follicles. Another study focused on cumulative ovulation rates in HMG-treated monkeys comparing a step-up and a step-down protocol (338). Most ovulations were found to occur before day 3 after hCG administration when the step-down protocol was used. However, additional follicles ruptured on days 4 and 5 when a step-up protocol

TABLE 3. Clinical outcome of low-dose step-up regimens for gonadotropin induction of ovulation (starting dose 1/2–one ampoule/day) in clomiphene citrate-resistant anovulatory infertile women, diagnosed as polycystic ovary syndrome

	Author, published year, and ref. no.							
	Seibel *et al.*, 1984 (323)	Buvat *et al.*, 1989 (324)	Hamilton-Fairley *et al.*, 1991 (325)	Dale *et al.*, 1993 (330)	Herman *et al.*, 1993 (331)	Balen *et al.*, 1994 (332)	Strowitzki *et al.*, 1994 (333)	Homburg *et al.*, 1995 (334)
Patient diagnosis	PCO	PCOS	PCOS	PCOS	PCOS	PCOS	PCOS	PCOS
No. of patients	5	23	100	50	30	103	20	25
No. of cycles	6	44	401	66	75	603	27	59
Ovulatory cycles (%)	66	70	72	74	96	68	93	79
Conceptions								
per started cycle (%)	[a]	16	[a]	18	[a]	14	26	17
per ovulatory cycle (%)	[a]	23	[a]	22	19	20	28	24
Cumulative pregnancy rate (%)	50	[a]	55	[a]	47	72	35	40
Multiple pregnancy rate (%)	[a]	0	4	[a]	21	18	28	0
Abortion rate (%)	[a]	14	32	33	[a]	16	0	30
Hyperstimulation rate (%)[b]	[a]	5	4	3	4	8	15	0

PCO, Polycystic ovaries; PCOS, polycystic ovary syndrome.
[a] No information provided.
[b] No uniform classification.

was applied, and it was concluded by the authors that follicle maturation was better synchronized resulting in a narrow 'ovulatory window' with a step-down protocol.

On the basis of physiological considerations (as discussed in *Section II.E*) and the above mentioned studies performed in the monkey, our group has focused on establishing a protocol for gonadotropin induction of ovulation applying decremental doses once ovarian response is established (so-called 'step-down' protocol) (169, 170). The major goal has been to design a safe and effective dose regimen for gonadotropin induction of ovulation that approximates physiological circumstances as closely as possible (173, 339–341). During initial studies only, patients were cotreated with a GnRH agonist in an attempt to reduce chances of interference of exogenously administered gonadotropins with unpredictable changes in endogenous FSH release. Using a regimen of two ampoules/day with two decreasing steps of one-half ampoule, daily blood sampling revealed that serum FSH levels showed a 2.1-fold increase followed by a subsequent decrease of 10% per day for 4 days (173), remarkably similar to previous studies in the monkey (143). Growth of follicles was sustained and ovulation achieved in the great majority of patients using this step-down regimen. The observed major variability in early follicular phase increase in serum E_2 (representing differences in the FSH threshold for stimulation of ovarian activity) was shown to predict chances for ovarian hyperstimulation. In a subsequent study (341) both immunoreactive and bioactive serum FSH concentrations were compared between step-down gonadotropin induction of ovulation and regularly cycling volunteers. Similar maximum follicular phase FSH concentrations were noted as well as similar late follicular phase daily FSH decreases. However, late follicular phase levels are lower due to a greater number of days of decreasing FSH levels during the normal menstrual cycle (median 7 *vs.* 4 days) (see also Fig. 11, and Table 4). It should be emphasized that daily blood samples were drawn 24 h after the previous injection. Pharmacokinetic studies of exogenous gonadotropins (336) revealed maximum FSH serum levels approximately 6–8 h after injection, and maximum levels were estimated to be approximately 30% higher as compared with 24 h concentrations. This opposes normal conditions where only minimal FSH changes during the day have been reported (342). In case of mono-follicle development during step-down gonadotropin protocols, growth rate and E_2 production by the dominant follicle is identical to those of the normal menstrual cycle (340).

Initial dose finding studies have generated a dose regimen that can be used in clinical practice. We have abandoned the use of GnRH agonists since 1992 without any loss of clinical efficacy. A similar FSH dose regimen is applied; *i.e.* a two-ampoule/day starting dose shortly after a spontaneous or progestagen-induced bleeding, followed by a decrease to one and one-half ampoules/day once a dominant follicle can be visualized by TVS (at least one follicle ≥ 10 mm). The dose is further decreased to one ampoule/day 3 days after the first dose reduction. See Table 5 for a summary of our clinical results in 234 cycles (343). Only one or two large preovulatory follicles were observed in 95% of stimulated cycles, and the median duration of treatment was 10 days (Fig. 12). Comparison of the group of women who did or did not conceive
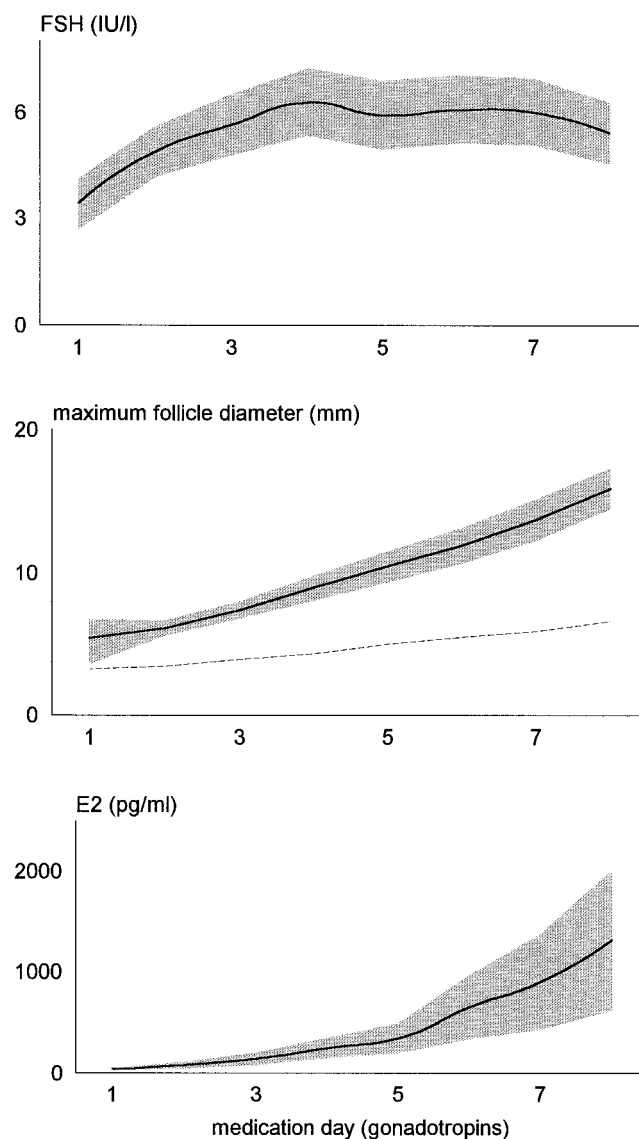
FIG. 11. Follicular phase serum FSH levels (*upper panel*), maximum follicle diameter (mm) (*middle panel*), and E_2 levels (*bottom panel*) (mean and 95% confidence intervals) according to medication day in 22 women with normogonadotropic anovulatory infertility during gonadotropin induction of ovulation using a step-down dose regimen. The *dotted line in the middle panel* indicates mean size of all observed follicles. Patients were pretreated for 3 weeks with a GnRH agonist (Buserelin; 1200 μg/day, intranasally) and medication was continued until hCG administration. The starting dose of gonadotropins was two ampoules (= 150 IU FSH)/day intramuscularly. The dose was decreased to one and one-half ampoules/day when at least one follicle ≥ 10 mm could be visualized, and to a final dose of one ampoule/day 3 days later. [Derived from Refs. 173 and 341.]

during treatment showed no significant differences with regard to body weight as well as initial serum LH and T levels, which appears to be different from observations using the low-dose, step-up regimen. The observed reduction in the duration of stimulation, as well as a lower total number of ampoules per stimulation cycle, may represent significant benefits in terms of health economics (reduced drug costs per cycle, possibly a reduced number of visits to the clinic, and more ovulations in a given time period). See Table 6 for a

TABLE 4. Endocrine and sonographic characteristics of the follicular phase during gonadotropin induction of ovulation using a decremental dose regimen, in 22 clomiphene-resistant normogonadotropic anovulatory women[#]

	Median (range)
Day 1 of medication	
FSH concentration (IU/liter)	3.6 (0.9–6.3)
E_2 concentration (pg/ml)	31 (12–72)
Follicle diameter (mm)	3 (2–4)
FSH	
Maximum	
Concentration (IU/liter)	7.6 (3.9–10.9)
Medication day	4 (1–9)
Minimum	
Concentration (IU/liter)	4.7 (2.7–8.2)
Medication day	7 (4–12)
Decrease	
IU/liter/day	1.5 (0.5–6.6)
Dominance (at least one follicle $\geq$ 10 mm)	
Cycle day	4 (2–7)
E_2 concentration (pg/ml)	296 (54–866)
Preovulatory (day of hCG administration)	
Follicle size (mm)	19 (18–21)
E_2 concentration (pg/ml)	488 (76–3016)
Medication day	8 (5–12)

In these studies patients were pretreated for 3 weeks with a GnRH agonist (Buserelin; 1200 μg/day, intranasally) and medication was continued until the day of hCG administration. HMG doses administered were two ampoules (= 150 IU FSH)/day as the starting dose, followed by decreasing daily doses of one and one-half ampoules/day (after dominance) and one ampoule/day (3 days later), intramuscularly. Blood sampling was performed daily. [Derived from Refs. 173 and 341.]

TABLE 5. Clinical outcome of step-down regimens for gonadotropin induction of ovulation (starting doses two to three ampoules/day) in clomiphene citrate-resistant normogonadotropic anovulatory infertile women

|	Author, published year, ref. no.	
	Mizunuma *et al.*, 1991 (345)	van Santbrink *et al.*, 1995 (343)
Patient diagnosis	PCOS	WHO II
No. of patients	9	82
No. of cycles	17	234
Ovulatory cycles (%)	100	91
Conceptions		
per started cycle (%)	29	16
per ovulatory cycle (%)	29	17
Cumulative pregnancy rate (%)	[a]	47
Multiple pregnancy rate (%)	20	12
Abortion rate (%)	[a]	19
Hyperstimulation rate (%)	[a]	2[b]

PCOS, Polycystic ovary syndrome; WHO II, World Health Organization, class II (defined as normal serum FSH and normal E_2 levels).
[a] No information provided.
[b] Mild cases only.

summary of major findings regarding the step-down protocol. It is too early to draw final conclusions regarding success and complication rates of this treatment modality. Clearly, only randomized comparative trails with sufficient statistical power can eventually determine whether the step-down approach represents a realistic alternative for every day practice of gonadotropin induction of ovulation.

Various other attempts pointing in the same direction have been published in the literature. Growth of the dominant
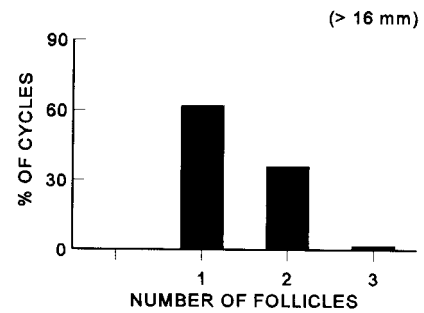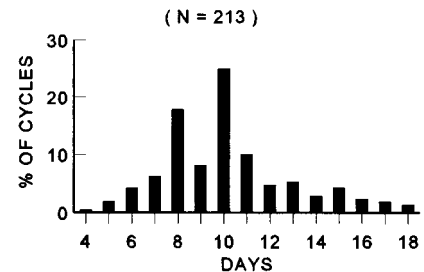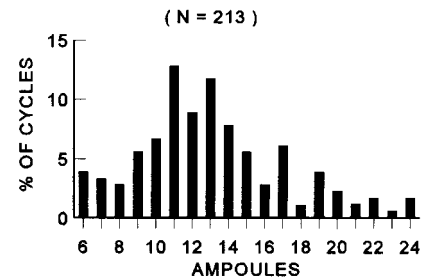
FIG. 12. Distribution of number of follicles larger than 16 mm (*upper panel*), duration of treatment (from day 1 of gonadotropin administration until 1 day after hCG injection) (*middle panel*), and required number of ampoules of gonadotropins per cycle (*bottom panel*) in 213 ovulatory cycles after gonadotropin induction of ovulation using a step-down dose regimen in 82 normogonadotropic clomiphene-resistant anovulatory women. In this clinical study no GnRH agonist comedication was used. [Adapted with permission from E. J. P. van Santbrink *et al.*: *Hum Reprod* 10:1048–1053, 1995 (343).]

follicle could be sustained in a woman with hypogonadotropic hypogonadism when exogenous FSH doses were decreased by 10% per day for 4 days after increased estrogen secretion (344). Clinical results of a step-down regimen in 22 PCOS patients, applying initial doses of three ampoules/day, with a reduction of the daily dose to one ampoule/day on day 3 (345) are also summarized in Table 5. Preliminary results (346) concerning a large multicenter study (involving 175 patients) comparing a step-up and a step-down regimen (initial dose three ampoules/day) in a prospective randomized fashion have been reported in abstract form. This study suggested a similar number of large preovulatory follicles comparing both treatment groups, a reduced preovulatory

TABLE 6. Summary of major findings regarding the step-down dose regimen (initial doses two ampoules/day, followed by two decreasing steps to one and one-half and one ampoule/day) for gonadotropin induction of ovulation

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FSH profile resembles FSH levels during the follicular phase of the normal menstrual cycle

Safe and effective clinical use has been established in an uncontrolled, single-center study

Short duration of treatment and low number of ampoules per stimulated cycle, is user friendly, and may suggest health economics benefits

Absence of apparent effects of obesity and elevated initial LH levels on treatment outcome

Major individual variability in ovarian response suggest differences in the FSH threshold. A starting dose of two ampoules/day is too high for some patients

Potential for further improvement of efficacy and safety

E_2 level in the latter group, and similar ovulation and cancellation rates. There were no differences in multiple pregnancy and hyperstimulation rates. However, for unknown reasons the pregnancy rate was significantly reduced after the step-down protocol. Unfortunately, this study was never reported in full detail, and the question whether the particular dose regimen used should be held responsible for observed differences remains unanswered. Preliminary results applying a sequential step-up and step-down regimen in 20 PCOS patients were reported recently (initial daily dose varied from one to one and one-half ampoules, and doses were subsequently reduced again to one ampoule daily after the leading follicle had reached a diameter of 14 mm) (347). As compared with step-up protocols, a significant reduction in late follicular phase E_2 levels and number of large and medium-sized follicles was observed. Another interesting approach is the use of sequential treatment with FSH (starting dose two ampoules/day), followed by pulsatile GnRH administration (20 μg pulses, subcutaneously, every 120 min) when the follicle reached a diameter of 11 mm. Results in 18 hypogonadotropic anovulatory patients were reported recently (348). Late follicular phase serum FSH levels were greatly diminished when pulsatile GnRH was applied, again resulting in a significant reduction in the number of large preovulatory follicles (1.3 *vs.* 3.9).

IV. Steroid Contraception and Residual Ovarian Activity

A. *The concept of follicle growth during partial and transient suppression of circulating FSH*

Oral contraceptives inhibit ovarian activity and ovulation through negative feedback actions of administered synthetic steroids on the hypothalamic-pituitary axis (349). The estrogen compound is believed to primarily inhibit FSH secretion, whereas progestins are supposed to mainly inhibit LH. Key effects of progestins involve reducing the frequency of the hypothalamic GnRH pulse generator (350). However, the contention of disparate effects of steroids on gonadotropin release has not been carefully investigated, and studies comparing the effects of estrogens alone *vs.* estrogen/progestin combinations on pituitary-ovarian function are lacking. Steroid contraception is well tolerated, exceptionally effective,

and extensively used worldwide. In an attempt to reduce side effects and to diminish the potential for short and long-term complications, estrogen doses have been gradually reduced. Since the introduction of oral contraceptives in the early 1960s, daily doses of ethinylestradiol (EE) in commercially available preparations have been diminished from 150 to 20 μg. Combined steroid pills with EE doses as low as 10 μg/day have proven effective when medication is taken correctly (351). Combined steroid contraceptives containing 1 mg of micronized estradiol have also been shown to inhibit ovulation, although control of bleeding was insufficient (352). In addition, novel, so-called second and third generation, progestins with reduced androgenic side effects have been developed and introduced in contraceptive regimens (353). Progestins may be combined with estrogens or may be administered alone. Progestin only (oral and depot) preparations have been tested extensively in recent years to provide women with the alternative of estrogen-free contraceptives. However, reduced suppression of pituitary FSH release introduced the need for continued progestin medication, which negatively affects cycle control.

Although pill effectiveness has not been compromised substantially, diminished suppression of circulating FSH by reduced steroid doses may give rise to substantial residual ovarian activity, as well as reduced tolerance for pill omission or for other circumstances that reduce circulating steroid concentrations (354–356). Follicle growth and concomitant E_2 production usually occur during the pill-free interval and the first week of pill intake, or when tablets are missed. Pill omission has been reported to occur in a substantial proportion of pill users in everyday practice (up to 27% of women in a 3-month period) (357, 358) and is clearly associated with contraceptive failure (359–363). In the great majority of studies published so far, monitoring of ovarian function is performed infrequently (screening intervals usually varies between twice weekly or once every month), and hormone assays and ultrasound for the assessment of follicle growth are rarely combined. However, substantial ovarian activity is uniformly reported when women use steroid regimens that are presently on the market. The concept arises that FSH levels rise during the pill-free interval above the 'threshold' for follicle recruitment (Fig. 6, *bottom panel*), and that follicle growth around the stage of dominant follicle selection is usually arrested after initiation of the next pill cycle. Improved understanding of ovarian activity during oral contraceptive medication may help to design novel strategies for steroid contraception.

B. *Ovarian suppression during steroid contraception*

1. Significance of initiation of pill intake and duration of treatment. According to some authors previous steroid medication does not seem to influence suppressive activity of combined steroid contraceptives (364, 365). Starting with the first pill cycle on a fixed day of the week, as initially advised, may postpone initiation of pill intake until day 6 of the normal menstrual cycle. If dominant follicle selection has already occurred (which may certainly be the case on cycle day 6, as discussed in *Section II.E.3*), progression of follicle growth may occur after pill intake (366). A careful study involving 58 sponta-

neous cycles and 22 first oral contraceptive pill cycles convincingly showed that a far greater suppression of ovarian activity was achieved by starting on day 1 as compared with day 5. As much as 60% (n = 11) of day 5 starters reached dominant follicle development (367). Hence, it should be advised that the first treatment cycle begin on cycle day 1 or 2 (368). Some investigators observed a trend toward a minor increase in ovarian activity during extended pill use (369). In contrast, gonadotropin suppression early in the pill cycle was reported to be similar over a 9-month treatment period (370). Few detailed studies have been published regarding the effect of previous use of oral contraceptives on follicular phase characteristics after stopping pill intake.

2. *Comparison of different steroid doses, compounds, and regimens.* Greater ovarian suppression was observed when pills containing 50 *vs.* 35 μg EE were compared (351, 371). Moreover, suppression of FSH was shown to be less pronounced in monophasics containing 20 μg/day EE as compared with 30 μg/day EE (372). Consequently, the margin for error is reduced if doses of daily steroid intake are diminished. According to some investigators, low-dose triphasic pills appear to be slightly less effective in ovarian inhibition as compared with monophasic preparations (373–375). Little difference, if any, was noticed when ovarian activity during intake of various progestational agents was compared (369, 374, 376, 377).

In contrast, follicle growth up to the preovulatory stage (378) and sonographic or endocrine evidence of ovulation have been reported in 30–60% of women in whom continued progestin-only pills or implants are used (379–384). Studies employing daily blood sampling showed major differences in luteal phase characteristics, but all with substantial follicular phase E_2 production (382) (Fig. 13). It has become apparent that the individual response to progestin-only medication is extremely variable. Substantial changes in serum E_2 levels coincide with follicle growth in these women. Surprisingly, some women exhibit extended periods with supraphysiological E_2 levels, whereas other women show complete ovarian suppression with serum E_2 levels in the postmenopausal range. Moreover, extended use of progestin-only implants may coincide with reduced suppression of ovarian activity (385). As opposed to combined contraceptives, positive feedback effects of endogenous E_2 may override negative feedback actions of exogenous progestins alone. However, the LH surge is usually blunted and progesterone may merely be secreted by luteinized follicles. Because pregnancy rates remain extremely low, it appears that other mechanisms such as luteal phase deficiency, and progestin effects directly on cervical mucus quality, endometrial and possibly tubal function prevent conception in these cases. Irregular bleeding is the major drawback, which prevents large-scale use of this contraceptive method (386). A connection between irregular bleeding and residual ovarian activity has been described (383). A discontinued progestin regimen combined with melatonin, the pineal hormone involved in seasonal breading in some animal species, has also been explored as a potential contraceptive agent (387). However, contraceptive efficacy of this regimen has never been demonstrated.

3. *Pill-free interval and pill omission.* The pill-free interval may impair contraceptive efficacy (355). However, potential benefits are the reassurance of monthly withdrawal bleeding, a lower monthly quantity of synthetic steroids, and improvement of metabolic changes during the pill-free week. A considerable rise in serum FSH levels and subsequent ovarian activity is usually observed during the pill-free interval and
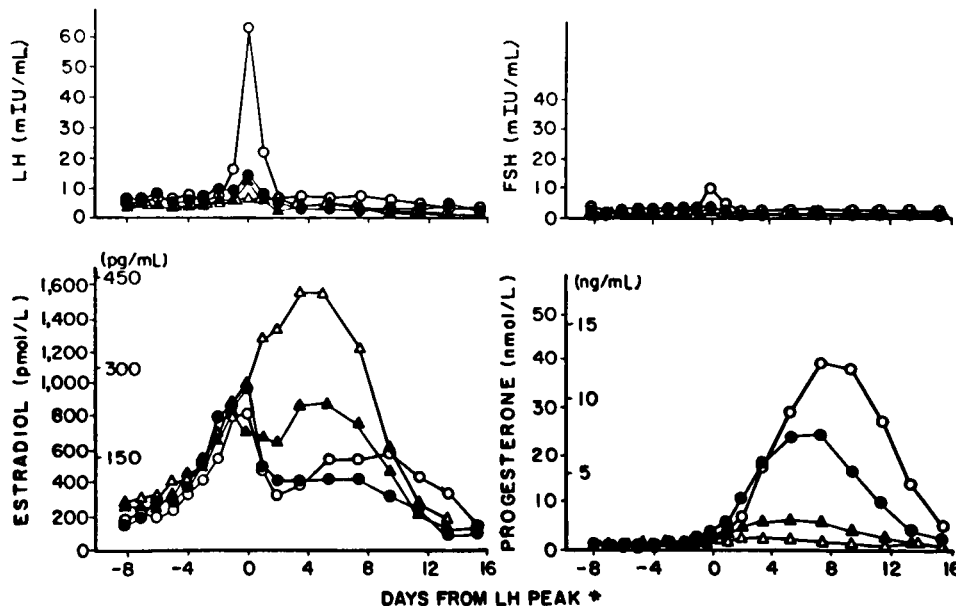
FIG. 13. Serum hormone (LH, FSH, estradiol, and progesterone) levels in regularly cycling controls (n = 12; ○-○) and depot progestagen (Norplant) users presenting with luteal activity (n = 12; ●-●), minimal luteal activity (n = 5;▲-▲), or no luteal activity (n = 14; △-△). [From Faundes A, Brache V, Tejada AS, Cochon L, Alvarez-Sanchez F. Ovulatory dysfunction during continuous administration of low-dose levonorgestrel by subdermal implants. *Fertil Steril* 1991; 56:27–31. Reproduced with permission of the publisher, The American Society for Reproductive Medicine (formerly The American Fertility Society).]

the first week thereafter (388–390). At the end of the pill-free week, integrated gonadotropin concentrations and pulse patterns (350) were indistinguishable from those of controls, whereas E_2 concentrations were significantly lower (389, 391). Ultrasound scanning on day 7 of the pill-free interval in 120 volunteers showed follicles more than 10 mm diameter in 23% of women (392). Frequent monitoring in 31 females showed that maximum E_2 concentrations usually occur on day 1 of the pill cycle, and maximum follicle diameter (median 10–12 mm) was observed on day 3 (369). These studies confirm indeed that maximum ovarian activity is present shortly after the pill-free week.

Studies from our own group (393) (A. M. van Heusden and B. C. J. M. Fauser; unpublished observations), applying daily blood sampling and TVS during the pill-free period in 36 low-dose, combined oral contraceptive pill users (containing daily doses of 50 μg EE and 2.5 mg lynestrenol) confirm that maximum FSH levels at the end of the pill-free interval are similar to early follicular phase maximum levels in the natural cycle [7.4 (4.1–12.8) *vs.* 6.6 (4.4–11.2) IU/liter]. Moreover, maximum E_2 levels during or shortly after the pill-free week are also comparable to E_2 levels at the day of dominant follicle selection in the normal cycle; 36 (8–85) *vs.* 47 (25–97) pg/ml. Twenty five percent of women exhibit follicles $\geq$ 10 mm. See also Fig. 14, and Tables 1 and 7.

It has appeared that the omission of one or more pills frequently occurs among oral contraceptive pill users. When transient interruption of several days of pill intake occurs in the middle of the pill cycle after a period of at least 7 days of uninterrupted use, the duration of reduced suppression of FSH release is not sufficient to allow for substantial reactivation of ovarian function (394). This observation, however, is not confirmed in all studies (390, 395), and contraceptive efficacy may still be compromised (396). In addition, the incidence of spotting was reported to be significantly increased in subjects omitting the pill in the second half of the pill cycle (394, 397). Ovulation and subsequent conception may take place when pill omission occurs around the pill-free interval. Various studies have been undertaken to investigate ovarian activity after the deliberate and transient interruption of pill intake during different phases of the pill cycle (398). Pill omission around the pill-free interval—effectively extending the period of pituitary recovery—results in more pronounced ovarian activity with continued growth of follicles (399).

C. *Follicle growth dynamics during contraceptive regimens*

Between 23 and 90% of combined oral contraceptive pill users have been reported to exhibit follicles beyond 10 mm diameter during frequent pelvic ultrasound (367, 369, 375, 388, 392, 400–402) (Fig. 15 and Table 8). Less information is available regarding accompanying serum E_2 levels. In about 25% of cycles, E_2 levels were reported to be above early follicular phase concentrations in control cycles (400). This should be considered stages beyond dominant follicle selection, based on findings of follicle size and concomitant steroid production during the spontaneous menstrual cycle (163, 206) (see also *Section II.E.3*). Follicles were reported to grow until a size of 12 mm in 35% of 75 treatment cycles.
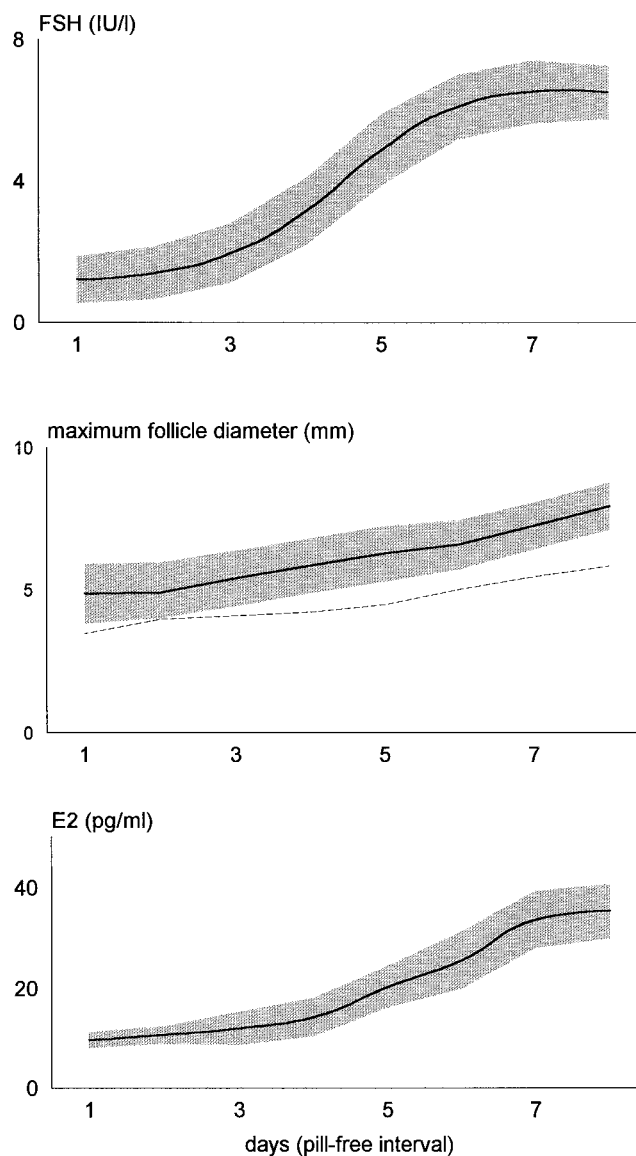
FIG. 14. Follicular phase serum FSH levels (*upper panel*), maximum follicle diameter (mm) (*middle panel*), and E_2 levels (*bottom panel*) (mean and 95% confidence intervals) according to day of the pill-free interval in 36 women using low-dose combined steroid contraceptive pills. Ultrasound scans and blood sampling were performed daily (A. M. van Heusden and B. C. J. M. Fauser). The *dotted line* in the *middle panel* indicates mean size of all observed follicles.

Follicles greater than 13 mm were rarely observed in some reports (388) but were seen frequently in other publications (400, 403). Preovulatory follicles ($\geq$ 18 mm) were observed in as much as 30% of cycles in a study involving 400 pill cycles (400). In a recent well designed study, follicles between 10 and 18 mm were observed in 30–50% of volunteers during treatment days 1–5 (403). Attempts to classify ovarian activity during oral contraceptive medication (404) have not gained wide acceptance. A biological rationale and appropriate reference values were lacking.

Once contraceptive pill intake is reinitiated, subsequent follicle maturation is usually arrested and follicles disappear (369). An elegant study was undertaken to monitor the ca-

TABLE 7. Endocrine and sonographic characteristics of the pill-free week in women using combined steroid contraceptive pills (n = 36)

	Median (range)
Day 1 of the pill-free week	
FSH concentration (IU/liter)	0.3 (0.1–7.8)
E_2 concentration (pg/ml)	8 (3–25)
Follicle diameter (mm)	5 (3–20)
Day 8 (first day of next pill cycle)	
FSH concentration (IU/liter)	5.9 (3.5–12.3)
E_2 concentration (pg/ml)	33 (5–83)
Maximum follicle diameter (mm)	8 (4–19)
Maximum FSH	
Concentration (IU/liter)	7.4 (4.1–12.8)
Day	7 (2–9)
Maximum E_2	
Concentration (pg/ml)	36 (8–85)
Day	8 (6–10)
Largest follicle	
Diameter (mm)	9 (6–21)
Day	9 (5–10)
Dominance	
(% of women with follicles $\geq$ 10 mm)	25

Medication involved low-dose combined steroid contraceptive pills. Sampling was performed daily for 10 days, starting on the first day of the pill-free week. [Derived from Ref. 393 and A. M. van Heusden and B. C. J. M. Fauser (unpublished observations).]
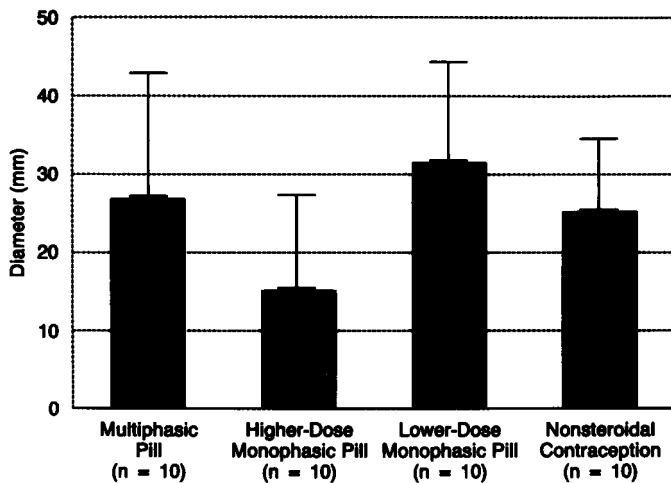

FIG. 15. Maximum diameter of the largest follicle (mean and SD) during six treatment cycles using various contraceptive regimens, such as multiphasic pills, higher-dose monophasic pills, lower-dose monophasic pills, and nonsteroidal contraception. [Reprinted with permission from The American College of Obstetricians and Gynecologists (*Obstetrics and Gynecology*, 1994, 83:29–34).]

pacity of these follicles to ovulate. Follicles were allowed to develop until their diameter reached 12 mm by extending the pill-free period by 3–7 days (405) (Fig. 16). Despite the initiation of contraceptive medication, most follicles continued to grow and responded to hCG administration by both rupture and luteinization. Hence, this study strongly suggests that follicles that reach a more advanced state of maturation during steroid contraceptive medication retain the capacity for continued growth and ovulation despite decreasing FSH levels. It has been suggested by some authors that the growth rate of the dominant follicle is slower as compared with normal conditions, whereas circulating E_2 levels remain normal according to follicle size (367). Incidentally, escape ovu-

lations have been observed during combined steroid contraception (400). However, this event rarely occurs with regular pill intake, because the endogenous LH surge does not occur even if follicles have reached full maturation (367, 388).

The occurrence of ovarian cysts in some patients may be related to preovulatory follicle development without subsequent ovulation. As described before, this is frequently observed during progestin-only medication. The occurrence of functional ovarian cysts may be prevented more effectively by monophasic (especially high-dose) pills as compared with triphasic combinations (406).

D. Alternative strategies for contraceptive development

Extensive breakthrough bleeding may occur in women using continued progestin-only contraception. This may be caused by endometrial atrophy, but may also be related to considerable individual variability in ovarian activity and subsequent estrogen production. Extended periods of E_2 levels above the normal range for regularly cycling women may also induce an increased risk for future health hazards.

During the pill-free interval FSH levels are reached that are in the same order of magnitude as during the early follicular phase of the normal menstrual cycle. Consequently, follicle recruitment takes place and dominant follicle selection occurs in a significant proportion of women using combined steroid contraception. The reduced need of the dominant follicle for continued support by high levels of FSH (see *Section II.E*) suggests that ongoing development until full maturation may occur despite decremental FSH serum levels due to the start of pill intake of the next cycle. Substantial follicle development has indeed been confirmed in contraceptive pill users. Unfortunately, uniform criteria to categorize ovarian activity during various steroid regimens have not been accepted, which renders it difficult to compare different studies. It has been documented that ovarian activity during similar contraceptive regimens vary widely (399, 407, 408). This observation may have important clinical implications because the magnitude of ovarian activity may be related to contraceptive efficacy, breakthrough bleeding, and ovarian cyst development. Tolerance for pill omission — especially early or late in the pill cycle, which effectively extends the pill-free interval — or for reduced availability of steroids (due to individual differences in steroid metabolism, drug interaction, diarrhea, or vomiting) has decreased substantially with the presently available low-dose contraceptives (355). Efficacy may be severely compromised in these cases. It should also be realized that tolerance for pill omission will be dependent on the developmental stage of follicles when pills are missed. On the basis of the above mentioned considerations, future strategies for further development of steroid contraceptive regimens (see also Table 9) may include:

1. The monthly 7-day pill-free interval has become critical for efficacy (especially tolerance for pill omission) of currently used low-dose contraceptives. A 7-day pill-free interval was arbitrarily chosen for reassurance to mimic the natural cycle, when the pill was introduced in the early 1960s. Doses may be diminished further or tolerance for error of

TABLE 8. Overview of studies focusing on residual ovarian activity during combined steroid contraceptive regimens

Author, published year (Ref.)	Study setting	Ultrasound observations (% of women with follicles $\geq$ 10 mm)	Endocrine observations
van der Vange, 1986 (400)	7 low-dose combinations (n = 70)	29% (17% follicles $\geq$ 18 mm)	27% serum E_2 levels > 75 pg/ml
Tayob *et al.*, 1990 (392)	Multiple combinations (n = 120)	23%	
Thomas and Vankrieken, 1990 (388)	30 μg EE + 75 mg GSD (n = 25)	up to 44%	No luteal activity
Grimes *et al.*, 1994 (375)	35 μg EE + NET mono-, triphasic (n = 45)	Between 29–50% (up to 13% ovarian cysts)	Up to 5% ovulation
Broome *et al.*, 1995 (378)	Triphasic; 30–40 μg EE + LNG (n = 17)	24%	
van der Does *et al.*, 1996 (369)	Triphasic; 30–40 μg EE + LNG or DSG (n = 31)	Up to 73% (up to 60% follicles > 15 mm)	1 ovulation, 1 LUF

EE, Ethinyl estradiol; GSD, gestodene; NET, norethisterone; LNG, levonorgestrel; DSG, desogestrel; LUF, luteinized unruptured follicle.
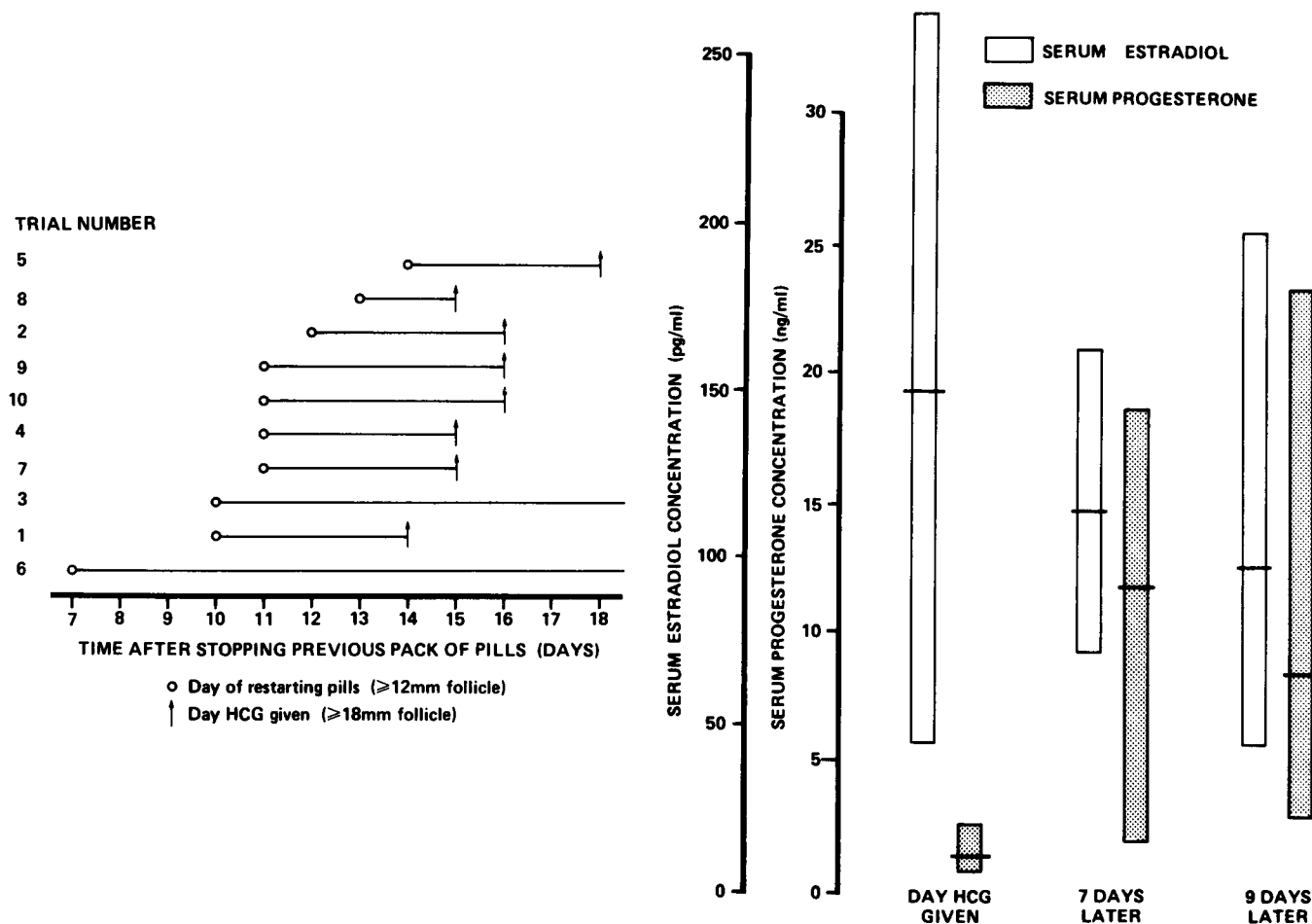
FIG. 16. The ovulatory potential of follicles was studied in 10 women by extending the pill-free interval (up to 7 additional days) allowing follicles to grow until 12 mm in diameter (◯). hCG was administered when follicles attained a diameter of 18 mm (*left panel*). Serum estradiol and progesterone levels (median and range) on the day of hCG administration and 7 or 9 days later (*right panel*) suggest normal ovulatory potential. [From Killick SR Ovarian follicles during oral contraceptive cycles: their potential for ovulation. *Fertil Steril* 1989; 52:580–582. Reproduced with permission of the publisher, the American Society for Reproductive Medicine (formerly The American Fertility Society).]

tablet taking may improve if the pill-free interval is reduced in length (*e.g.* to 5 days), or reduced in frequency (*e.g.* once every 2–3 months instead of monthly). Indeed, it has been shown that withdrawal bleeding can be extended to once every 2 (409, 410) or 3 (411) months in the majority of women, and it was stated (411) that 'many of the women who vol-

unteered liked using a contraceptive that also reduced the frequency of their menstrual periods.'

2. Steroid dose regimens of combined contraceptives may also be altered. It is uncertain whether there is anything to be gained by the presently available triphasic regimens. The most pronounced suppression of FSH is needed early in the

TABLE 9. Hypothetical options for reduction of residual ovarian activity and, therefore, improved efficacy of combined steroid contraceptive pills

1. Reduction of the duration of the 7 day pill-free interval or reduction of the frequency of the pill-free week
2. Alternative dose regimens, with higher doses of steroids early in the pill cycle
3. Continued estrogen intake during the pill-free interval
4. Assessment of individual ovarian sensitivity for FSH stimulation (so-called FSH threshold), possibly at the end of the pill-free interval, and adjustment of steroid doses on an individual basis

pill cycle, and therefore higher doses of steroids may need to be applied during the first days rather than later in the pill cycle. Testing of a decremental steroid dose regimen should perhaps be considered, although it is uncertain whether bleeding control would be compromised.

3. Low doses of estrogens could be continued during the pill-free interval without interference with bleeding patterns, as has been shown for postmenopausal hormone replacement therapy. This may reduce the rise in FSH and subsequent recovery of ovarian activity during the pill-free week.

Altogether, the above mentioned attempts are aiming at reducing the frequency, magnitude, or duration of pituitary recovery and subsequent initiation of gonadotropin-dependent follicle growth.

4. A completely different strategy focuses on the major individual variability in residual ovarian activity during similar steroid contraceptive medication. A 2.5-fold difference in maximum circulating FSH concentrations has been observed by our group in a carefully selected group of volunteers with normal ovarian function (163). This seems to indicate that a major individual variability in ovarian sensitivity for FSH stimulation exists. The observed variability in ovarian suppression during oral contraceptive medication may be related to a differences in ovarian 'FSH threshold,' rather than differences in sensitivity of the hypothalamic-pituitary unit for negative steroid feedback. It may be possible to predict which subject will be prone to escape ovulation and reduced contraceptive efficacy, by the individual assessment of hormone levels and follicle dynamics during contraceptive medication, particularly at the end of the pill-free interval. This approach may lead to better tailoring of dose and type of steroid regimens according to individual needs, providing an extended strategy for 'designer' (412) contraceptive pills.

V. Conclusions and Future Directions

Growth of follicles from the resting primordial stage until the preovulatory phase takes several months. Only the last 2 weeks of this long trajectory are dependent on stimulation by gonadotropins and can therefore be manipulated in the human at present. If maturing antral follicles achieve a distinct stage of development they are programmed to die. Only if serum FSH levels surpass a threshold (which is different from one individual to the other) these follicles are rescued from atresia, *i.e.* gain gonadotropin dependence, and continue their development. Under normal conditions, increased FSH levels above the threshold occur during the luteo-follicular transition. Subsequent decremental FSH con-

centrations during the follicular phase are crucial for single dominant follicle selection.

Continued growth of the dominant follicle despite reduced late follicular phase stimulation by FSH suggests increased sensitivity. Local autocrine up-regulation by increased intraovarian E_2 production has been implemented as the underlying cause of this reduced need for FSH stimulation. However, increased E_2 levels are only associated with follicle diameter exceeding 10 mm. Moreover, from this size onward a dominant follicle can be visualized by TVS, suggesting that only dominant follicle development is associated with increased aromatase enzyme activity in granulosa cells. Several lines of evidence have convincingly demonstrated that increased intrafollicular E_2 biosynthesis is not mandatory for continued follicle growth up to the preovulatory stage. These observations strongly, although indirectly, suggest that intraovarian modification of FSH takes place through other factors, and that as yet unidentified factors drive growth of the dominant follicle. The concept of a reduced need for stimulation by FSH of the dominant follicle bears significance for both gonadotropin induction of ovulation (*i.e.* stimulation of ovarian function by exogenous FSH in anovulatory infertile patients) and residual ovarian activity during low-dose steroid contraceptive regimens.

Gonadotropins have been used worldwide for over three decades for the treatment of anovulatory patients. Treatment is successful, although complication rates related to multiple follicle development remain high. Over the years, tools to monitor ovarian response have improved considerably and new drugs have been introduced. In addition, treatment regimens have been modified by lowering the doses administered. However, information regarding patient diagnosis and precise follow-up of the interplay between circulating FSH concentrations and follicle growth dynamics remains scarce. It may be possible to further improve the balance between success and complications by more rigidly applying physiological concepts. Endocrine profile and follicle growth during step-down FSH treatment compare almost precisely to changes observed during the follicular phase of the normal menstrual cycle.

Presently available, low-dose steroid contraception is characterized by extensive residual ovarian activity and reduced tolerance for omission of pill intake. The endocrine profile and follicle growth dynamics in pill users during and shortly after the pill-free interval are compared with the normal menstrual cycle. Alternative strategies for contraceptive development to improve the safety margin can be postulated on the basis of this comparison.

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