

## Lack of correlation between maximum early follicular phase serum follicle stimulating hormone concentrations and menstrual cycle characteristics in women under the age of 35 years

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**The gradual increase in follicle stimulating hormone (FSH) concentrations in women approaching menopause results from the depletion of the ovarian follicular pool, a process referred to as 'ovarian ageing'. This study investigates whether variable endogenous FSH concentrations, as have been observed in normo-ovulatory young women, are related to menstrual cycle characteristics, including predictors of ovarian ageing. Serum concentrations of immunoreactive FSH, oestradiol, and inhibin-A and inhibin-B were measured, and follicular growth was assessed by transvaginal ultrasound throughout the follicular phase in 39 healthy volunteers (20–35 years) with regular menstrual cycles. Median serum FSH concentration on cycle day 3 was 5.1 IU/l (range 3.6–11.2), and median maximum follicular phase FSH was 6.2 IU/l (range 4.3–11.2), observed on cycle day 6 (range 2–15). Maximum FSH concentrations were not correlated with age or cycle length, nor with maximum inhibin-B. The number of small (<10 mm) antral follicles on cycle day 3 was 11 (range 4–21) and was not correlated with age, nor with maximum FSH. Inhibin-A remained low until a significant rise on cycle day 9 (range 3–12), which was significantly correlated with the late follicular rise in oestradiol ( $r = 0.56$ ,  $P = 0.01$ ). These observations indicate a lack of correlation between maximum follicular phase serum FSH concentrations and parameters of ovarian ageing in women under the age of 35 years. In addition, FSH concentrations assessed on cycle day 3 represent an underestimation of maximum early follicular phase FSH. Distinct individual differences in intra-ovarian modification of FSH action, resulting in differences in the FSH threshold for stimulation of ovarian function, may be operative.**

**Key words:** FSH/inhibin-A/inhibin-B/menstrual cycle/oestradiol

### Introduction

In women approaching menopause, endocrine and menstrual cycle changes indicate altered ovarian function. The most consistent endocrine finding is elevated early follicular phase serum follicle stimulating hormone (FSH) concentrations,

which are not accompanied by a rise in luteinizing hormone (LH) (Sherman *et al.*, 1976; Ahmed Ebbiary *et al.*, 1994). This gradual increase in FSH concentrations may result from decreased endocrine feedback signals through diminished production of oestradiol and inhibins (Sherman and Korenman, 1975; Klein *et al.*, 1996a) by the decreasing follicular pool (Richardson *et al.*, 1987; Faddy *et al.*, 1992). Recently, the number of antral follicles in the early follicular phase, as assessed by ultrasound (Reuss *et al.*, 1996) and peripheral levels of one of the inhibins, inhibin-B (Klein *et al.*, 1996b), has been found to decrease with advancing age, which may reflect a reduction in the number of recruited follicles. The remaining reproductive potential has been referred to as 'ovarian reserve' (Scott and Hofmann, 1995). Evaluation of ovarian reserve prior to initiation of ovarian stimulation by measuring basal or stimulated FSH concentrations in the early follicular phase may provide prognostic information regarding chances for success of infertility treatment (Navot *et al.*, 1987; Scott *et al.*, 1989; Fanchin *et al.*, 1994; Scott and Hofmann, 1995; Hansen *et al.*, 1996; Kim *et al.*, 1997).

Throughout reproductive life, increased FSH concentrations during the luteo-follicular transition of the menstrual cycle stimulate growth of a cohort of small antral follicles (Hall *et al.*, 1992). According to the threshold concept, the inter-cycle rise in FSH should surpass a distinct level in order to recruit this cohort of follicles (Brown, 1978). Later during the follicular phase, one of the recruited follicles will gain dominance and eventually ovulate (Hodgen *et al.*, 1985). Concomitant with the presence of a dominant follicle, serum oestradiol concentrations increase, whereas FSH concentrations decrease (van Santbrink *et al.*, 1995). Apart from oestradiol, inhibin-B secreted in the early follicular phase by the cohort of recruited growing follicles may also be involved in the negative feedback regulating FSH secretion (Groome *et al.*, 1996).

A recent study from our group showed distinct (up to 2.5-fold) differences in maximum serum FSH levels in a well-defined group of young women presenting with normal ovarian function (van Santbrink *et al.*, 1995). This observation may be related to distinct differences in the FSH threshold level due to different intra-ovarian modification of FSH action by growth factors (Seifer *et al.*, 1995; Fauser and van Heusden, 1997). Therefore, high FSH concentrations may not necessarily indicate decreased ovarian reserve. The aim of the present study is to examine to what extent the variation in endogenous FSH stimulation of the ovary is related to menstrual cycle characteristics in young normo-ovulatory women.

## Materials and methods

### Subjects and study design

This study was approved by the local Ethics Review Committee and written informed consent was obtained from all participants. Volunteers were acquired through advertisement in a local newspaper and were paid for their participation. A total of 39 women with regular cycles entered the study. Inclusion criteria for this study were: age 20–35 years, a history of regular menstrual cycles for at least 6 months with cycle lengths between 26 and 31 days, normal body weight [body mass index (= weight divided by the square of body length) between 19 and 24 kg/m<sup>2</sup>], no medical or hormonal treatment during at least 3 months prior to the study, and no previous history of infertility.

Daily blood withdrawal was performed between 13.00 and 17.00 h, starting several days before the expected onset of menses until the next ovulation. Transvaginal sonography of the ovaries was performed every other day, from the initiation of the study until sonographically assessed ovulation (disappearance or >50% decrease in size of largest follicle, if >15 mm), as described previously by Pache *et al.* (1990) and van Santbrink *et al.* (1995). Six or 7 days after ovulation a blood sample was taken to assess mid-luteal progesterone levels.

### Hormone assays

Blood samples were centrifuged (10 min at 2500 r.p.m.) within 2 h after withdrawal and serum was stored at –20°C until assayed. FSH and LH levels were assessed by immunoradiometric assays. Some of the samples (from 16 subjects) were assayed using the assay obtained from Medgenix (Fleurus, Belgium), as described previously by Fauser *et al.* (1991). Samples from the remaining 23 subjects were assayed with the Amerlite assay (Ortho-Clinical Diagnostics, Amersham, UK). Comparative analysis of both assays revealed no significant differences between the results obtained using both methods (Amerlite = 1.00 × Medgenix – 0.02 U/l;  $r = 0.93$ ,  $n = 20$ ). Intra- and interassay coefficients of variation were <3% and <8% for FSH, and <5% and <15% for LH, respectively. Oestradiol levels were assessed by radioimmunoassay provided by Diagnostic Products Corporation (Los Angeles, CA, USA), with intra- and interassay coefficients of variation <15% and <18%, respectively (van Santbrink *et al.*, 1995). Mid-luteal progesterone was determined by radioimmunoassay as described by de Jong *et al.* (1974), with intra- and interassay coefficients of variation being <16% and <17%, respectively. Inhibin-A and inhibin-B levels were measured using an enzyme-linked immunosorbent assay obtained from Serotec (Brooks University, Oxford, UK) as described earlier by Groome *et al.* (1996). Intra- and interassay coefficients of variation were <9% and <15%, for both assays respectively. Inhibin-A and inhibin-B levels were measured in serum samples obtained every other day, taking the day of maximum FSH levels (FSH<sub>max</sub>) as reference. Dependent on individual cycle length, these samples had been collected between 8 days prior to day of FSH<sub>max</sub> and 8 days thereafter. In all hormone assays each sample was tested in duplicate, with all samples from one subject in the same assay.

### Data analysis

Results are presented as mean ± SD if normally distributed, and as median and range if distributed otherwise. Correlation coefficients given are Spearman's or Pearson's, dependent on the distribution. *P*-values are two-sided with 0.05 taken as the limit for statistical significance. Day of dominance is defined as the day at which the dominant follicle was 10 mm (assessed by transvaginal sonography), and was determined by extrapolating the linear growth curve of the pre-ovulatory follicle backward until the day on which the diameter

of this follicle was 10 mm, as described previously by Pache *et al.* (1990) and van Santbrink *et al.* (1995). Day of oestradiol rise was estimated using piece-wise linear regression relating log(oestradiol) to cycle day as described previously by van Santbrink *et al.* (1995). Maximum FSH concentration was defined as the highest level of FSH during the follicular phase, disregarding the post-nadir increase prior to the LH peak.

## Results

### Cycle characteristics

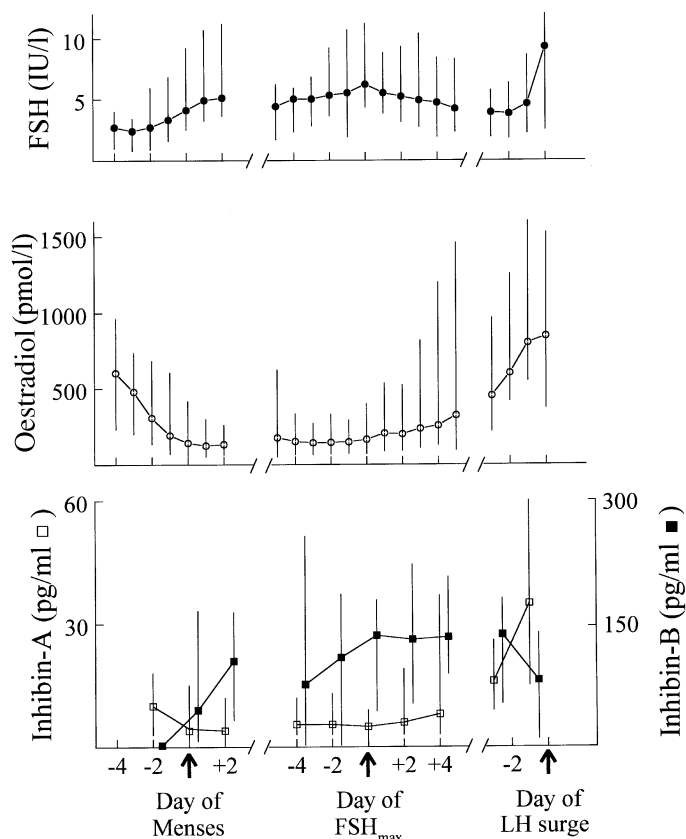
The mean age of the subjects studied was 28 ± 4 years. All 39 subjects were ovulatory during the studied cycle as demonstrated by transvaginal sonography and by elevated mid-luteal progesterone levels (41 ± 14 nmol/l). Median cycle length was 28 days (range 24–31 days). In 5 subjects the study cycle was of shorter duration than expected on the basis of cycle history. The mean duration of the follicular phase was 15 ± 3 days, and of the luteal phase 13 ± 2 days. In this population, age was not correlated significantly with cycle length, nor with the length of the follicular phase ( $r = -0.08$ ,  $P = 0.61$  and  $r = -0.29$ ,  $P = 0.08$ , respectively) (data not shown).

### Hormone concentrations

Median levels and range of FSH, oestradiol and inhibin-A and inhibin-B during the follicular phase are depicted in Figure 1. The median FSH concentration on cycle day 3 was 5.1 (3.6–11.2) IU/l, and did not correlate with age ( $r = 0.19$ ,  $P = 0.24$ ). During the follicular phase, FSH levels reached a maximum level of median 6.2 (4.3–11.2) IU/l on cycle day 6 (2–15). The distribution of the FSH concentrations on cycle day 3 and on the day of maximum FSH concentration (FSH<sub>max</sub>) is depicted in Figure 2. Maximum FSH levels did not correlate with age, cycle length or duration of the follicular phase (Figure 3).

On cycle day 1 the serum oestradiol concentration was 140 (70–420) pmol/l and remained at this level until cycle day 8 (4–15) when a log-linear increase occurred, as calculated with piece-wise linear regression. The cycle day at which the oestradiol concentration rose correlated significantly with the cycle day at which follicle dominance was assessed by transvaginal sonography ( $r = 0.70$ ,  $P < 0.01$ , data not shown). The correlation between day of FSH<sub>max</sub> and day of oestradiol rise was weak ( $r = 0.34$ ,  $P = 0.03$ , data not shown), while the correlation between day of FSH<sub>max</sub> and day of follicle dominance was not significant ( $r = 0.28$ ,  $P = 0.08$ , data not shown). Neither the day of oestradiol rise, nor the day of follicle dominance correlated with the FSH<sub>max</sub> concentration (Figure 4). After the day of oestradiol rise, oestradiol concentrations increased with a doubling time of 3.9 (2.1–5.4) days until a median pre-ovulatory level of 847 (370–1530) pmol/l was reached.

Inhibin-A and inhibin-B levels on cycle day 1 were 7 (3–18) pg/l and 40 (1–140) pg/l, respectively. Inhibin-A remained at this low level until cycle day 9 (3–12), when levels started to increase until 35 (15–88) pg/l at the day of LH surge. The cycle day of the inhibin-A increase was correlated with the

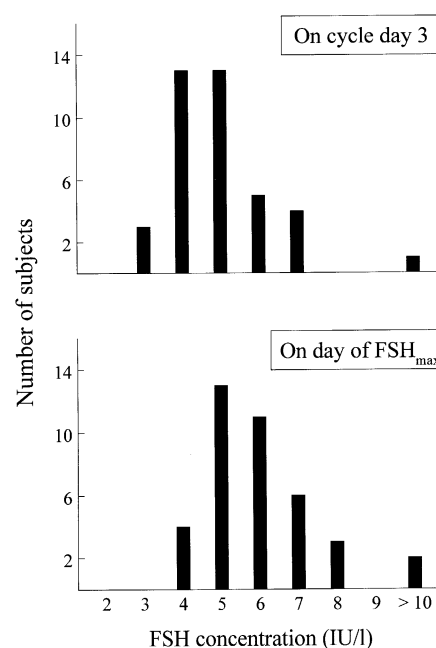


**Figure 1.** Follicle stimulating hormone (FSH) (top panel), oestradiol (middle panel) and inhibin-A (M) and inhibin-B (N) (bottom panel) serum concentrations during the follicular phase in 39 young, normo-ovulatory women. Data are presented as median values with bars spanning the 5th to 95th percentile. The time-scale on the x-axis is split into days around the onset of menses (day of menses = cycle day 1), around the day of maximum FSH concentrations (day of FSH<sub>max</sub>) and days prior to ovulation (day of LH surge).

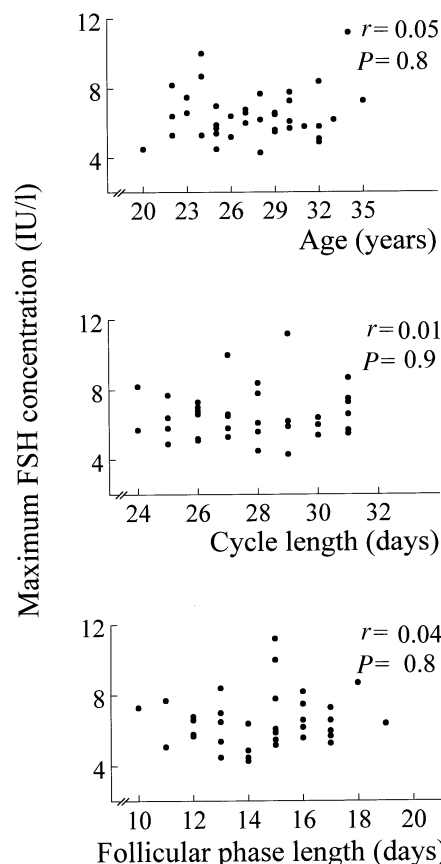
day of oestradiol rise ( $r = 0.56$ ,  $P < 0.01$ ; data not shown). Maximum inhibin-B levels were 170 (94–310) pg/l on cycle day 7 (1–14), decreasing thereafter to 77 (10–149) pg/l on cycle day 9 (2–15). The cycle day of maximum inhibin-B was correlated significantly with the day of FSH<sub>max</sub> ( $r = 0.53$ ,  $P < 0.01$ ; data not shown), whereas the maximum inhibin-B concentration was not correlated with FSH<sub>max</sub> ( $r = 0.05$ ,  $P = 0.80$ ), nor with age ( $r = -0.13$ ,  $P = 0.47$ , Figure 5).

**Follicle growth**

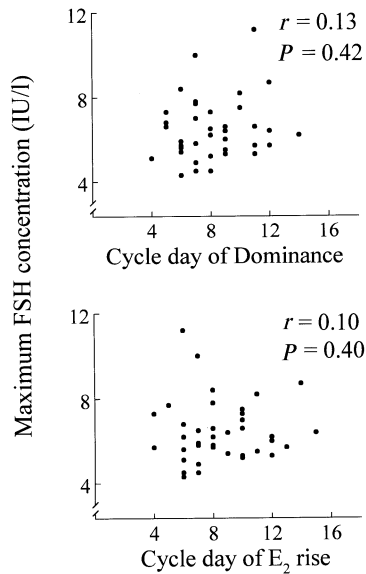
On cycle day 3 the mean number of small (2–10 mm) antral follicles was 11 (4–21) for both ovaries. The number of follicles in the early follicular phase was not correlated with FSH<sub>max</sub> ( $r = -0.10$ ,  $P = 0.70$ ), nor with age ( $r = -0.11$ ,  $P = 0.58$ ) (Figure 6). The number of antral follicles on cycle day 3, day of FSH<sub>max</sub> [13 (6–20)] or day of maximum inhibin-B [13 (6–22)] was not correlated with inhibin-B concentrations on these respective days ( $r = -0.20$ ,  $r = 0.07$  and  $r = 0.25$ , respectively; data not shown). However, the sum of the surface of each antral follicle present on a particular day (considered representative for the total inhibin-B producing capacity) was significantly correlated with the inhibin-B concentration ( $r = 0.40$ ,  $P < 0.01$ ) (data not shown).



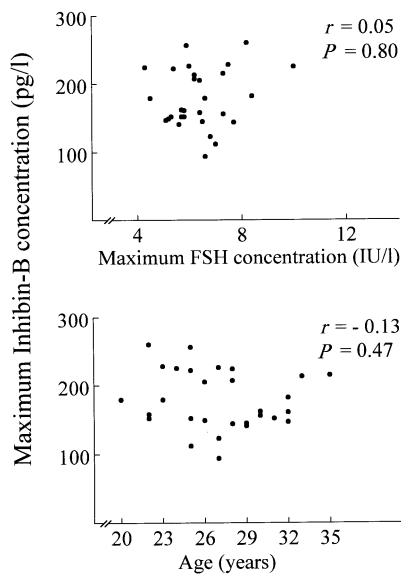
**Figure 2.** Distribution of follicle stimulating hormone (FSH) concentrations on cycle day 3 (top panel) and on the day of maximum FSH concentration (bottom panel), on the basis of daily blood sampling during the follicular phase in 39 young, normo-ovulatory women.



**Figure 3.** Distribution of maximum follicular phase FSH serum concentrations related to age (top panel), cycle length (middle panel) and length of the follicular phase (bottom panel) in 39 young, normo-ovulatory women. Correlation coefficients  $r$  are Spearman's.



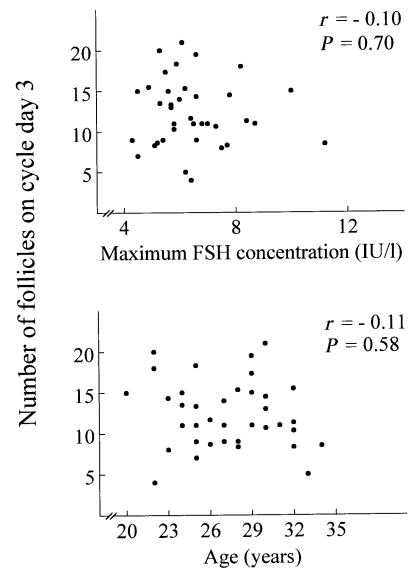
**Figure 4.** Distribution of follicular phase maximum serum follicle stimulating hormone (FSH) concentrations related to cycle day of dominance (as assessed by transvaginal sonography) (top panel) and cycle day of oestradiol ( $E_2$ ) rise (bottom panel) in 39 young, normo-ovulatory women. Correlation coefficients  $r$  are Spearman's.



**Figure 5.** Distribution of follicular phase maximum serum inhibin-B concentrations related to maximum follicle stimulating hormone (FSH) concentration (top panel) and age (lower panel) in 39 young, normo-ovulatory women. Correlation coefficients  $r$  are Spearman's.

After the day of follicle dominance [cycle day 7 (4–13)], the dominant follicle displayed a mean linear increase in size of  $1.5 \pm 0.4$  mm/day, until a mean pre-ovulatory size of  $20.5 \pm 3.0$  mm. The growth rate of the dominant follicle was not correlated significantly with the doubling time of oestradiol ( $r = -0.13$ ,  $P = 0.4$ ), nor with the doubling time of inhibin-A ( $r = -0.3$ ,  $P = 0.19$ ; data not shown). Both the growth rate of the dominant follicle and the pre-ovulatory size were not correlated to  $FSH_{max}$  ( $r = -0.1$ ,  $P = 0.72$  and  $r = -0.1$ ,  $P = 0.45$ , respectively; data not shown).

Despite the lack of correlation between the absolute hormone

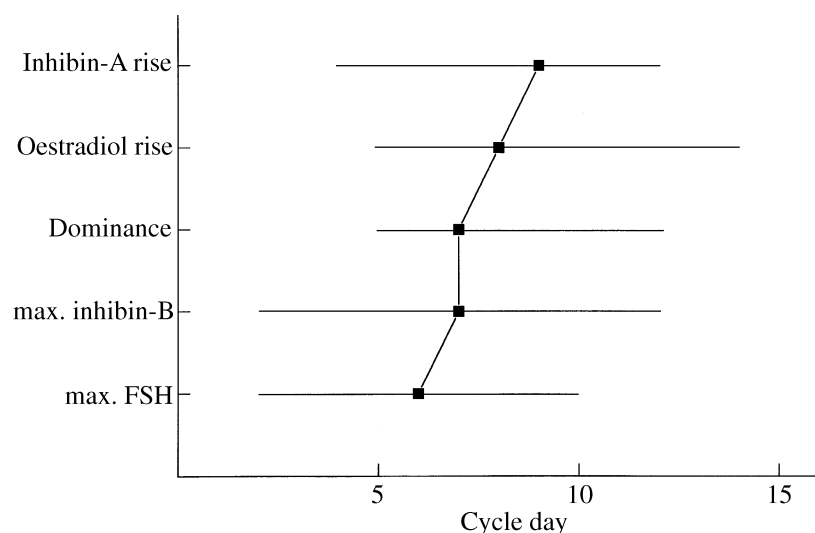


**Figure 6.** Distribution of the number of small antral follicles (2–10 mm) observed in both ovaries on cycle day 3 by transvaginal ultrasound related to maximum FSH concentration (upper panel) and age (lower panel) in 39 young, normo-ovulatory women. Correlation coefficients  $r$  are Spearman's.

concentrations, the data indicate a sequential order in the occurrence of day of  $FSH_{max}$  (median cycle day 6), day of maximum inhibin-B (median cycle day 7), day of follicle dominance (median cycle day 7), day of oestradiol rise (median cycle day 8) and day of inhibin-A rise (median cycle day 9) (Figure 7). The day of  $FSH_{max}$  occurred earlier in the cycle compared with that of maximum inhibin-B concentration ( $P < 0.01$ ), whereas the day of maximum inhibin-B concentration preceded the day of follicle dominance ( $P < 0.01$ ). No difference was found between day of selection, oestradiol rise and inhibin-A rise. The sequence of events as represented in Figure 7 is confirmed by a mean slope of  $0.89 \pm 0.57$  days ( $P < 0.01$ ) from day  $FSH_{max}$  until day of inhibin-A rise. Disregarding the days of  $FSH_{max}$  and maximum inhibin-B in the analysis revealed a mean slope of  $0.46 \pm 1.35$  days ( $P = 0.1$ ).

## Discussion

In the years prior to menopause early follicular phase serum FSH levels start to rise concomitant with changes in menstrual pattern (Treloar *et al.*, 1967; Ahmed Ebbiary *et al.*, 1994; Reame *et al.*, 1996). The diminished response of the ovary to FSH stimulation with advancing age is generally attributed to the decrease in quantity and quality of the pool of recruitable follicles (Richardson *et al.*, 1987; Meldrum, 1993). Eventually the follicular pool is exhausted and endocrine feedback mechanisms in the ovarian-hypothalamic-pituitary axis become disrupted, resulting in an increase in gonadotrophin concentrations to post-menopausal levels. It is assumed that, in the years preceding menopause, serum FSH levels indirectly reflect a woman's residual ovarian function, i.e. the ovarian reserve (Scott and Hofmann, 1995). Indeed, increased early follicular phase FSH concentrations in women of advanced reproductive



**Figure 7.** Cycle day at which maximum serum follicle stimulating hormone (FSH) concentration, maximum serum inhibin-B concentration, follicle dominance (as assessed by transvaginal sonography), oestradiol concentration increase and inhibin-A concentration increase occurred during the follicular phase in 39 young, normo-ovulatory women. Data are presented as median values with bars spanning the 5th to 95th percentile.

age (>35 years) have been associated with decreased success of infertility therapy (Muasher *et al.*, 1988; Scott *et al.*, 1989; Ebrahim *et al.*, 1993; Cahill *et al.*, 1994; Hansen *et al.*, 1996). Assessment of ovarian reserve prior to infertility treatment by measuring FSH serum levels on cycle day 3 has been put forward as a good predictor of treatment outcome.

Results from the present study show that, in normo-ovulatory young women, FSH concentrations on cycle day 3 span a wide range between 3.6 and 11.2 IU/l. Furthermore, the maximum FSH concentrations during the follicular phase displayed a comparably wide range of 4.3 to 11.2 IU/l, although at a higher median level. For each individual, FSH concentrations displayed an increasing trend from cycle day 1 onwards until the day on which a maximum FSH concentration was reached with a median of cycle day 6 (range 2–15). FSH serum concentrations on cycle day 3 are clearly an under-representation of maximum follicular phase serum concentrations. Owing to shortening of the follicular phase, this discrepancy may be reduced in older women.

The observed large inter-individual difference in maximum FSH concentrations suggests a variation in ovarian sensitivity for FSH stimulation in normo-ovulatory women. However, this is not reflected in a wide variation in ovarian function, since relevant biological outcome parameters such as follicle development and oestradiol production appear not to be related to the height of the maximum FSH concentration. It was not possible to demonstrate a significant correlation between early follicular phase FSH concentrations and chronological age or to confirm the relationship between increased FSH concentrations and a relative shortening of the menstrual cycle as a consequence of a diminished ovarian function, as has been observed in women approaching menopause (Treloar *et al.*, 1967). Furthermore, results from the present study do not indicate a significant correlation of the number of antral follicles during the early follicular phase with maximum FSH concentrations or with age, although it has been reported

recently that the number of antral follicles during the follicular phase decreases with advancing age (Reuss *et al.*, 1996). An accelerated decrease in follicle number has been observed after the age of 37 years (Faddy *et al.*, 1992), which is beyond the age of the subjects in the present study.

Over 20 years ago it was proposed that increasing FSH concentrations in women approaching menopause might be due to diminished inhibin production by the ovaries (Sherman *et al.*, 1976). More recently, with the advance of specific immunoassays for inhibin-A and inhibin-B, it has been shown that serum inhibin-B concentrations increase in the early follicular phase in response to increasing FSH concentrations, whereas inhibin-A concentrations increase at a later stage during the cycle, reaching a maximum concentration in the mid-luteal phase (Groome *et al.*, 1996). Apart from a negative feedback effect at the level of the pituitary regulating the secretion of FSH, the presence of inhibin-B in the follicular fluid of the largest, dominant follicle suggests a local, intra-ovarian role for this protein in follicle selection (Groome *et al.*, 1996). Recently, it has been demonstrated that low (<45 pg/ml) inhibin-B concentrations in the early follicular phase are related to a reduced response to ovulation induction and indicate decreased ovarian reserve, irrespective of age (Seifer *et al.*, 1997). Inhibin-B concentrations are believed to represent the size of the cohort of follicles recruited for further development. The observed lack of correlation between maximum FSH and maximum inhibin-B concentrations in the present study suggests indirectly that the number of follicles recruited for further development is independent of the magnitude of stimulation by FSH.

Results from the present study and from a previous study by our group (van Santbrink *et al.*, 1995) demonstrate large inter-individual variation in follicular phase FSH concentrations in normo-ovulatory young women. Although all subjects showed monofollicular growth, apparently governed by a similar pattern of hormonal events, as shown in Figure 7, differences in maximum FSH concentrations did not correlate

with parameters characteristic of ovarian ageing, such as menstrual cycle changes, and endocrine and ultrasound observations. The height of the FSH concentration may represent differences in sensitivity of the ovary to FSH stimulation (i.e. the FSH threshold), which in turn may be determined by a variety of intra-ovarian factors (Fauser, 1996). The significance of a single serum FSH determination for assessment of ovarian ageing may be reduced because a large overlap exists between younger and older subjects. This may reflect physiological differences in the FSH threshold. Changes in early follicular phase serum FSH concentrations in a given individual over time could provide a more sensitive test of ovarian ageing (te Velde *et al.*, 1997). Differences in local enhancement and regulation of FSH action by intra-ovarian factors such as insulin-like growth factors or the activin and inhibin system may be involved (Giudice *et al.*, 1996). This concept is strongly supported by recent observations showing differences in follicular fluid insulin-like growth factor-II concentrations as a function of day 3 serum FSH in women independent from age (Seifer *et al.*, 1995). In addition, individual differences in the circulating FSH isohormone profile, differing in metabolic clearance rate and in-vitro or in-vivo bioactivity, may also be important (Ulloa-Aguirre *et al.*, 1995). Finally, the possibility of individual differences in FSH receptors cannot be excluded.

The general perception is that high follicular phase serum FSH concentrations indicate advanced reproductive ageing and reduced outcome of infertility therapy. Data presented in the present study suggest that large individual differences in FSH occur in normo-ovulatory women under the age of 35 years and that other factors not related to ovarian ageing, such as differences in ovarian sensitivity to FSH, should also be considered.

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**I.Schipper et al.**

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