# Subtle disorders of the menstrual cycle in subfertile and aging women

Van Zonneveld, Pieter
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Universiteit Utrecht

# Subtle disorders of the menstrual cycle in subfertile and aging women

Subtiele stoornissen van de menstruele cyclus
bij verminderd vruchtbare en ouder wordende vrouwen

(Met een samenvatting in het Nederlands)

**Proefschrift**

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door

**Pieter van Zonneveld**

geboren op 14 februari 1948 te Leiden

| | |
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| Promotor: | Prof. dr. E.R. te Velde |
| Co-promotoren: | Dr. F.J.M. Broekmans |
| | Dr. H.P.F. Koppeschaar |

Beoordelingscommissie: Prof. dr. M.A. Blankenstein

Prof. dr. H.W. Bruinse

Prof. dr. B.C.J.M. Fauser

Prof. dr. C.J.M. Lips

The patient is the centre of the medical
universe around which all our work revolves
and towards which all our efforts tend
G.B. Murphy 1857-1916

Voor Marie-Anne
Voor Floris, Fleur, Mirit en Jasmijn
Voor mijn moeder
Aan de nagedachtenis van mijn vader

# Contents

# Chapter 1

INTRODUCTION, PART 1

## Chapter 1. Introduction, part 1

Oligomenorrhoea, generally defined as a mean cycle length >6 weeks, and amenorrhoea, less than one menstrual period in 6 months, are frequent causes of subfertility or infertility, since they are present in about 20% of the couples that attend a fertility clinic (Hull *et al.*, 1985). Regular cycles, especially when they are accompanied by symptons such as premenstrual breast-swelling or dysmenorrhoea, are usually regarded as ovulatory. Progesterone production in the second half of the cycle further indicates the occurrence of ovulation. Progesterone may be demonstrated qualitatively by recording a basal body temperature chart, as well as quantitatively by measuring progesterone concentrations in blood or saliva. However, since ultrasound techniques have been developed by which the growth of ovarian follicles, and the occurrence of ovulation can be visualized, several subtle disorders have been described in women with regular menstrual cycles (Coutts *et al.*, 1982; Polan *et al.*, 1982; Kerin *et al.*, 1983; Daly *et al.*, 1985; Hamilton *et al.*, 1985; 1987; Lewinthal, 1986; Eissa *et al.*, 1987; Petsos, 1987; Van Zonneveld *et al.*, 1994; Rodin *et al.*, 1994). Ovulation may either not occur at all (luteinized unruptured follicle, LUF) or may occur too early. (Polan *et al.*, 1982; Eissa *et al.*, 1987). In LUF cycles a normal increase of serum estradiol concentrations is observed as well as a normal increase in diameter of the dominant follicle. However, in spite of the fact that progesterone levels rise to (almost) normal levels during the luteal phase, the follicle does not rupture. Ovulation more than 48 hours after the LH peak value is considered to occur too late (World Health Organization, 1980; Eissa *et al.*, 1986; O'Herlihy *et al.*, 1980; Zegers-Hochschild *et al.*, 1984, Rodin *et al.*, 1994). Much attention has been paid to a disorder called luteal phase defect (LPD), defined as an insufficient development of the endometrium to permit a pre-embryo to implant. LPD is assumed to be caused by a shortage of progesterone production by the corpus luteum or an inadequate response of the endometrium to normal progesterone levels. However, whether LPD really is a cause of subfertility has not been established (Li and Cooke, 1991). Little is known about the course of LH serum levels during the menstrual cycle. Elevated levels during the follicular phase have been found in patients who had recurrent miscarriages compared to controls (Regan *et al.*, 1990), but a beneficial effect of strategies by which LH levels can be lowered, has not

been demonstrated (Balen *et al.*, 1993). The pattern of the LH surge has been related to the probability of conception in spontaneous cycles in which insemination was applied because of cervical hostility. The pregnancy rate was significantly higher in cycles with an LH surge of 2 days compared to cycles with a surge of 1 day. Whether a suboptimal LH surge results from a less optimal follicular phase, or from an insufficient positive feedback system, is unknown (Cohlen *et al.*, 1993).
In elderly but still regularly cycling women, the first endocrine change by aging is a monotropic rise of early follicular FSH (Sherman *et al.*, 1976; Musey *et al.*, 1987; Lee *et al.*, 1988; Fitzgerald *et al.*, 1994; Ahmed Ebbiari *et al.*, 1994; Klein *et al.*, 1996; Reyes *et al.*, 1997). Shortening of the follicular phase of the cycle is another characteristic of reproductive aging (Treloar *et al.*, 1967; Lenton *et al.*, 1984; Klein and Soules, 1998). Elevated early follicular FSH levels have been found in regularly cycling women with unexplained subfertility (Ahmed Ebbiari *et al.*, 1994; Leach *et al.*, 1997). The authors postulate that this may represent a very early sign of follicle depletion. Elevated FSH levels have also been described in premenopausal mothers of hereditary dizygotic twins. This was attributed to an altered responsiveness of the hypothalamus and/or pituitary to ovarian factors, not to age (Lambalk *et al.*, 1998). Whether disorders of follicle growth or ovulation occur more often in aging women who still have regular cycles, is unknown.

In this chapter we will first describe characteristics of the normal menstrual cycle. Subsequently, we will summarize the present knowledge on subtle cycle disorders and their relation with subfertility. In Chapter 2, we will focus on the age-related decline of fertility.

## The normal menstrual cycle

The three major organs involved in the regulation of reproductive function are the hypothalamus, the pituitary gland (the so-called central system) and the ovaries. For many years it has been thought that the cyclical pattern of the menstrual cycle is governed by the central system via the gonadotropins that stimulate the ovarian follicles. However, it has become clear that the follicle system of the ovary provides the key signals for the ovulation, the develop-

ment of the corpus luteum, and the timing of menstruations (Nakai *et al.*, 1978; Knobil, 1980).

## The central system

Within the arcuate nucleus of the hypothalamus, neuro-endocrine cells produce the gonadotropin-releasing hormone (GnRH), a decapeptide. These neurons are influenced by neurotransmitters from other parts of the brain, and hormones, mainly originating from the ovaries. Gonadotropin-releasing hormone reaches the anterior pituitary through the portal vessels located in the pituitary stalk, and stimulates the synthesis and release of the glycoproteins follicle stimulating hormone (FSH) and luteinizing hormone (LH) by the same gonadotropin-secreting cells, the so-called gonadotrops. Differences in FSH and LH responses are caused by different feedback effects of ovarian hormones. To produce FSH and LH the gonadotrops require a pulsatile stimulation by GnRH (Knobil, 1980), while constant or excessive GnRH stimulation causes their desensitization. During the early follicular phase of the menstrual cycle the pulse interval is about 90', increasing to about 60' during the late follicular phase. In the luteal phase the pulse frequency slows down to an interval of some hours. FSH and LH consist of two amino-acid chains (a- and b-subunits) which combine to a heterodimer. Apart from FSH and LH the a-subunit is present in human chorionic gonadotropin (hCG) and thyroid stimulating hormone (TSH). The b-subunit is unique for each hormone and determines the specific biologic activity.

## The ovary

From 4-6 weeks of gestation the primordial gonads can be identified in human embryos as ridges, created by the coelomic surface epithelial cells. The germ cells are thought to migrate from the yolk sac to the gonadal ridges, where they start to multiplicate. At 16-20 weeks of gestation the ovaries are estimated to contain 7 million oogonia (Baker, 1963). From about that time a decrease of the stock of germ cells starts. The oogonia are transformed to oocytes by entering the prophase of the first meiotic division after which they are arrested in the last stage of the prophase, and surrounded by one layer of flat

granulosacells, the combination of which is called a primordial follicle. The oocytes stay in this arrested stage for many years, and only resume meiosis in the dominant follicle just before ovulation. After birth the loss of primordial follicles continues, leaving only about 300,000 of them at the menarche. Menopause occurs when almost the entire stock of follicles is exhausted.

## Regulation of the menstrual cycle

For an understanding of the processes that take place, the menstrual cycle can be divided into 3 phases: the late-luteal and follicular phase, the peri-ovulatory phase and the luteal phase.

### *The late luteal and early follicular phase*

From the store of primordial follicles, some follicles start to grow continuously by a process of maturation. Such development of resting follicles is considered to be a gonadotropin-independent process, although there are indications for a facilitating effect of FSH (Te Velde *et al.*, 1998). After several months of growth a group of follicles emerges that have matured to a stage in which they can be stimulated by FSH to further growth. From this cohort of small antral follicles, one follicle will be selected which eventually ovulates , the so-called leading or dominant follicle. The selection of the dominant follicle from the cohort of small antral follicles , during the late luteal and early follicular phase, lasts not more than 10 days (Gougeon, 1996). As a consequence of the continuing maturation of primordial follicles there is always a number of small antral follicles that can be stimulated to further growth, provided FSH levels exceed a certain threshold-level during a sufficient period of time, the so-called FSH window (Brown, 1978; Fauser and Van Heusden, 1997). It is assumed that the most advanced and most sensitive healthy follicle is the one that will become the dominant follicle (Glasier *et al.*, 1989). The other follicles of the same cohort are destined to undergo atresia by a process of programmed cell death.
A late luteal and early follicular rise in FSH blood levels is the result of lysis of the corpus luteum at the end of its life-span. This lysis causes a declining secretion of steroid hormones ($E_2$ and progesterone) and protein substances

(e.g. inhibin A), resulting in a diminished negative feedback upon the central system. (Nippold *et al.*, 1989; Le Nestour *et al.*, 1993; Danforth *et al.*, 1998; Welt *et al.*, 1999; Santoro *et al.*, 1999). A subsequent increase in GnRH pulse frequency is followed by an increase of FSH and -to a lesser degree- of LH (Hall *et al.*, 1992). Under the influence of FSH the granulosacells of the preantral follicles are able to aromatize androgen precursors into estrogens. Androgens are synthesized in the theca cells by stimulation of LH, and reach the granulosacells by diffusion through the basement membrane (the so-called two-cell, two-gonadotropin system) (Kobayashi *et al.*, 1990). In cooperation with estrogens, FSH also stimulates an increase in the number of its own receptors (LaPolt *et al.*, 1992), and the proliferation of granulosa cells (Yong *et al.*, 1992).
As the follicles develop, follicular fluid accumulates between the granulosa cells forming a a space, the antrum. The granulosa cells that surround the oocyte differentiate into cells called cumulus cells. Increasing production of estrogens and probably also inhibin B result in decreasing FSH levels by negative feedback (Glasier *et al.*, 1989; Van Santbrink *et al.*, 1995; Groome *et al.*, 1996). The dominant follicle is thought to survive by its enhanced sensitivity for FSH due to its superior amount of FSH receptors, while less developed follicles do not withstand the fall in FSH (Glasier *et al.*, 1989; Fauser and van Heusden, 1997) .

*The peri-ovulatory phase*

In concert with estradiol FSH induces the development of LH receptors on granulosa cells of large follicles. (Kessel *et al.*, 1985; Hillier *et al.*, 1985). As estradiol rises, a positive feedback mechanism towards the central system is activated. As a result, a transition from suppression to stimulation of LH occurs, and its massive release, the so-called LH-surge (Hoff *et al.*, 1983). An untimely start of the LH surge is thought to be prevented by a gonadotropin surge-inhibiting factor (GnSIF), secreted by the ovary (De Koning, 1995). LH induces luteinization of the granulosa cells, which start the production of progesterone. The LH surge is considered as the most reliable predictor of ovulation, which occurs 34-36 h after its onset and 10-12 h after its peak level (Hoff *et al.*, 1983). The LH surge stimulates several events: 1. The resumption

of the first meiotic division, 2. Luteinization of granulosa cells, 3. Expansion of the cumulus cells, which enables the oocyte to be released at ovulation, 4. Synthesis of substances including prostaglandins and proteolytic enzymes, that are necessary to weaken the wall of the follicle and enable its rupture.

*The luteal phase*

After ovulation, luteinization continues. Granulosa cells enlarge and accumulate a yellow pigment, lutein. The so-called corpus luteum is formed. Theca cells also luteinize and become part of the corpus luteum. An extensive angiogenesis takes place, induced by LH and growth factors. Large amounts of progesterone and $E_2$ are produced, reaching a maximum at about 8 days after ovulation. For its normal lifespan of about 14 days the corpus luteum requires (small amounts of) LH (Vande Wiele *et al.*, 1970). During the luteal phase gonadotropin levels are low due to negative feedback by the combined actions of $E_2$, progesterone and also inhibin A. Luteolysis starts 9-11 days after ovulation, but the mechanisms controlling this process are not yet clear. The life-span of the corpus luteum can only be prolonged by hCG, that is produced by a developing pregnancy. Otherwise, declining $E_2$ and progesterone result in shedding of the endometrium and menstruation.

## Criteria to consider a cycle as normal

The development of a dominant follicle determines the length of the follicular phase, while the length of the luteal phase remains more or less stable. The length of the follicular phase and thus the length of the menstrual cycle shortens gradually from age 30 until about 45. Some years before menopause the mean cycle length increases again due to the presence of anovulatory cycles and irregular bleeding (Treloar, 1967).
Although much is known about hormonal patterns and follicle development in the normal menstrual cycle, it is difficult to define cycle abnormalities unless there is no follicle growth at all or no ovulation. In some studies criteria for normality have been defined (Van Zonneveld *et al.*, 1994). It has been suggested that conception cycles are the gold standard for normality, however, reports on large series are lacking. In addition, also in such 'ideal' cycles,

hormone levels and sonographic findings vary greatly. For example, ovulation of a follicle of 13 mm may result in a normal pregnancy (Van Zonneveld *et al.*, 1994). Moreover, the occurrence of pregnancy is dependent on many female and male factors and therefore it is likely that, on the one hand, many 'ideal' cycles never result in a pregnancy, e.g. because of suboptimal timing of intercourse, whereas, on the other hand, women with suboptimal cycles may conceive, provided all other contributing factors are perfect. The occurrence of pregnancy is the result of a complex and multifactorial process, and it is well possible, therefore, that the achievement of clear-cut and generally applicable criteria of cycle normality is an impossible goal.

Follicular growth has been studied including conception and nonconception cycles. Table 1 shows the pre-ovulatory follicle diameters, that have been found in conception cyles; in Table 2, follicle diameters before ovulation or follicle aspiration for in-vitro fertilization (patients with a spontaneous LH surge) are shown. O'Herlihy *et al.* (1980) found a pre-ovulatory range of mean follicular diameter of 17-25 mm, and a good correlation between the calculated volume on the basis of ultrasound, and the volume of aspirated follicular fluid. Other groups found similar pre-ovulatory follicle diameters. Zegers-Hochschild *et al.* (1984) found in 13 spontaneous conception cycles a variation of 15-23.3 mm (Table 1) with a 95% interval of 18.3-21.0 mm; Eissa *et al.* (1986) a range of 18-25 mm in 12 spontaneous conception cycles. From ovulation induction studies it is known, that the diameter is related to their probability to ovulate. Stanger and Yovitch (1984) found that 80% of follicles larger than 16 mm at the time of HCG ovulated, in contrast to 27% of follicles of 15-16 mm in diameter. This was confirmed by Silverberg *et al.* (1991) who observed that follicles of 17 mm or larger are more likely to ovulate after HCG administration than smaller follicles. In their IVF laboratory, Bomsel-Helmreich *et al.* (1987) could not find nuclear maturation in oocytes that were collected from follicles smaller than 16 mm in diameter. Follicle rupture (Table 3) is assumed if the follicle has disappeared or markedly decreased in size (Hamilton *et al.*, 1985; Thomas and Cooke, 1988). Fluid in the pouch of Douglas, being confirmatory evidence, is not always seen. Eissa *et al.* (1986) observed a decrease in diameter of at least 89% in 9 conception cycles, within 48h after the LH peak value. They consider a decrease in mean follicle diameter of at least 60% as evidence of ovulation (Eissa *et al.*, 1987).

**Table 1.** Pre- ovulatory follicle diameters in conception cycles

| Author | Group characteristics | No. of cycles | Characteristics |
|---|---|---|---|
| Zegers-Hochschild *et al.*, 1984 | 25 multiparous volunteers | 13 cycles | Follicle diameters 15-23.3 mm (mean 19.65) |
| Eissa *et al.*, 1986 | Spontaneous cycles in infertility patients | 12 cycles | In 1 cycle no follicle visible; in 11 cycles foll. diam. 18-25 mm (mean 19.4 mm) |
| | Ovulation induction cycles (several methods) | 8 cycles | Follicles 15.7 - 21.3 mm (mean 17.8 mm) (note: hCG administered in some cycles) |

**Table 2.** Pre-ovulatory follicle diameters, or diameters before follicle aspiration in cycles with a spontaneous LH surge

| Author | Group characteristics | No. of patients and cycles | Follicle diameters |
|---|---|---|---|
| O'Herlihy *et al.*, 1980 | IVF cycles | 36 patients; 29 spontaneous, 7 stimulated cycles | 17-25 mm after the start of the LH surge |
| Zegers-Hochschild *et al.*, 1984 | Non-conception cycles in 25 multiparous volunteers | 15 cycles | 15.6-25.5 mm (mean 19.1) before ovulation |
| Eissa 1986 | Spontaneous cycles in infertility patients | 12 cycles | In 1 cycle no follicle visible<br>In 11 cycles follicle diameter 18-25 mm (mean 19.4 mm) |

**Table 3.** Definition of ovulation by ultrasound

| Author | Definition or observation |
|---|---|
| Zegers-Hochschild *et al.*, 1984 | Observed disappearance of echo-free structure in all of 28 cycles |
| Hamilton *et al.*, 1985 | Disappearance or a decrease in mean follicular diameter |
| Lewinthal *et al.*, 1986 | Disappearance or decrease in size |
| Thomas and Cooke, 1988 | Disappearance or markedly decrease in size |
| Eissa *et al.*, 1986 | At least 60% decrease in diameter |
| Janssen-Caspers *et al.*, 1986 | At least 5 mm decrease in diameter |
| Vermesh *et al.*,1987 | Observed disappearance of the dominant follicle in 14 spontaneous cycles in infertility patients |
| Petsos *et al.*, 1987 | Disappearance or collapse within 48h after the LH surge |
| Check *et al.*, 1990 | At least 5 mm decrease in diameter, 3 days after hCG in HMG-stimulated cycles |
| Rodin *et al.*, 1994 | Observed >50% shrinkage or disappearance within 48h after the LH surge |
| Leach *et al.*, 1997 | Follicular collapse, >15% decrease in follicular size, and/or the presence of increased echogenicity within the follicle. |

Janssen-Caspers *et al.* (1986) postulated a decrease in diameter with 5 mm or more as evidence for ovulation. This was later confirmed by Check *et al.* (1990) in HMG-stimulated cycles. A lesser than 5 mm decrease resulted in a 5.4% and a 4% pregnancy rate in first and second induction cycles, respectively, compared to 13.5 and 15.7% when the decrease exceeded 5 mm. If no decrease at all was observed, no pregnancies followed. Pierson *et al.* (1990) visualized the evacuation of follicle content sonographically in 9 cycles. Although a great variation was seen, all follicular fluid had disappeared after a maximum of 21 minutes. In case no follicle rupture is evident by daily ultrasound examinations, no pregnancies were reported by Eissa *et al.* (1987) in 60 cycles, by Hamilton *et al.* (1987) in 30 cycles, and by Check *et al.* (1992) in 168 cycles. This supports the assumption that in these cycles no ovulation occurs. However, good imaging of the ovaries is a prerequisite. Eissa *et al.* (1987) described a pregnancy in a cycle showing no follicle at all on ultrasonography. After ovulation, the corpus luteum may appear as a cystic structure, as observed by Geisthövel *et al.* (1983) at 2,6 ± 5 days after disappearence of the follicle. This indicates that daily observations are necessary to establish whether or not ovulation has occurred.

Very few data are available on E2 levels. By some authors 400 pmol/l at the end of the follicular phase is considered to be the lower limit of normal (Lenton *et al.,* 1982; Thomas and Cooke, 1988).

Concerning progesterone values in the normal cycle, Hull *et al.* (1982) found midluteal progesterone values in conception cycles between 27 - 53 nmol/l, and between 3 - 80 nmol/l in nonconception cycles. This suggests not only a minimum level, but also an 'optimal range' for fertility. In their study, no visualization of the ovaries was used. Lenton *et al.* (1982) compared hormonal parameters in 14 conception cycles with 27 non-conception cycles. Higher progesterone levels were observed in the early and midluteal phase of the conception cycles, but some influence of the very early pregnancy itself could not be excluded. Hamilton *et al.* (1987) more often observed LUF cycles if progesterone remained under the 32 nmol/l level.

The lower limits of cycle normality as suggested in the literature, are summarized in Table 4.

**Table 4.** Minimal criteria for a normal cycle based on data from the literature

| |
|---|
| - Estradiol (E2) rise > 400 pmol/l (Lenton *et al.*, 1982; Thomas and Cooke, 1988) |
| - Mean follicular diameter 17 mm or more at the LH peak level (O'Herlihy *et al.*, 1980; Zegers-Hochschild *et al.*, 1984; Eissa *et al.*, 1986; Silverberg *et al.*, 1991) |
| - Ovulation: at least 60% decrease of mean follicular diameter (Eissa *et al.*, 1986) or decrease of at least 5 mm (Janssen-Caspers *et al.*, 1986; Check *et al.*, 1990) or 'disappeared or markedly decreased in size' (Thomas and Cooke, 1988; Hamilton *et al.*, 1985) |
| - Ovulation within 48 hours after the LH peak value (WHO, 1980; Hoff *et al.*, 1983; Eissa *et al.*, 1986) |
| - Serum progesterone at least 32 nmol/l in the luteal phase (Hull *et al.*, 1982; Hamilton *et al.*, 1987) |

## Subtle cycle disorders including luteinized unruptured follicle (LUF) cycles, as a cause of subfertility

In 1978, Marik and Hulka, as well as the group of Koninckx (Koninckx *et al.*, 1978), published their classical work on so-called luteinized unruptured follicle (LUF) cycles: cycles in which no ovulation stigmata could be seen at laparoscopic inspection of the ovaries, after ovulation was presumed to have occurred, followed by corpus luteum formation and progesterone production. Koninckx *et al.* (1978) suggested that LUF cycles could not be distinguished from ovulatory cycles by hormonal parameters. They found ovulation stigmata in only 10 of 24 patients with otherwise unexplained infertility. This may

well have been an overestimation, as re-epithelisation of the ovulation opening appeared to take place much earlier than was supposed. Scheenjes *et al.* (1990) showed that the process of closure starts already after 1.5 days following ovulation. Therefore, data obtained by laparoscopic visualization of an ovulation stigma, should be interpreted with caution. Hamilton *et al.* (1985), using ultrasound, described LUF cycles with a typical pattern of follicular growth (continuing growth after the LH surge instead of disappearance) in combination with a significant reduction of progesterone serum levels in the luteal phase, as well as LH peak values lower than those seen in control cycles. Normal follicular growth and normal hormonal levels were present until the LH peak. Ovulation, however, did not occur, and in most cycles progesterone levels remained below 32 nmol/l. Eissa *et al.* (1987) called such cycles 'cyst-like cycles' and they reserved the term 'LUF cycle' for cycles in which no rupture of the dominant follicle occurred, but instead of continuing growth, a decrease in follicle diameter after the LH surge was seen. In their concept of LUF, normal luteal phase progesterone levels are present. Other authors do not make the same distinction, and use the phrase 'LUF' for both disorders of ovulation. Eissa *et al.* (1987) described another abnormal pattern, which they called 'asynchronous cycles'. In these cycles a shrinkage of the dominant follicle occurs 24 h before the LH surge, without ovulation after the LH surge. The same phenomenon has been described by Polan *et al.* (1982), Lewinthal *et al.* (1986) and Rodin *et al.* (1994). They observed follicles that remained relatively small (< 16 mm) and luteinized before the onset of the LH surge. These follicles did not ovulate after the LH surge. Petsos *et al.* (1987) found a shrinkage of follicles before the LH surge, as well as large cyctic follicles which luteinized after the LH surge, without rupture. Daly *et al.* (1985) described small follicles that ovulated after the LH surge. In our opinion, any cycle in which growth of a dominant follicle is present till the LH surge, and after which luteinization occurs, but no ovulation, could be considered as a LUF cycle. However, in the literature the phrase 'LUF' is used to describe follicles that do not rupture after reaching a diameter within the normal pre-ovulatory range. To avoid confusion, we adapted this view in our review of the literature. Hamilton *et al.* (1987) observed 10 of 14 (71%) LUF cycles if midluteal progesterone did not reach 32 nmol/l, in contrast to 10 of 127 (7,9%) LUF cycles when progesterone was 32 nmol/l or more. Eissa *et al.* (1987) found as

many as 48 out of 89 (54%) abnormal cycles, including 19 'LUF' cycles (21%) and 21 'cyst formation cycles' (24%) in a group of 45 subfertile women. In contrast, in a recent publication Leach *et al.* (1997) found no abnormalities of follicle growth or ovulation in 12 cycles of 12 women who met rigid criteria for unexplained subfertility. In Table 5, the incidences of subtle cycle disorders, as diagnosed by several authors using ultrasound in spontaneous cycles, are summarized.

Anovulatory cycles like LUF, in which a pregnancy cannot occur, may represent the most extreme subtle cycle disorder. A pregnancy did not occur during 30 LUF cycles described by Hamilton *et al.* (1987), as was the case in 60 LUF cycles found by Eissa's group (1987) and 168 LUF cycles by Check *et al.* (1990). It is speculative whether other disturbances like rupture of small follicles, or low luteal phase levels of progesterone may represent cycles in which a pregnancy can occur, but is less likely. The publications, summarized in Table 5 include a total of 618 spontaneous cycles, in which a subtle cycle abnormality has been found in 143 (23%).

Recurrence of subtle cycle disorders has only been studied in a small number of patients (Table 6). Eissa *et al.* (1987) described 22 normal first cycles that were followed by 11 normal and 11 abnormal cycles; 23 abnormal first cycles were followed by 14 abnormal and 9 normal cycles. They also found different types of abnormalities when they followed subsequent cycles in the same individual. Kerin *et al.* (1983), found a low incidence of recurrence (in one of eight women with a LUF cycle). Hamilton *et al.* (1985) found recurrence of LUF cycles in 6 patients. Two of them had a history of pelvic inflammatory disease (PID), three received ovulation-induction treatment and and one patient, who was treated with ovulation induction, had a PID in the past. Do LUF cycles also occur in healthy women who have regular cycles?. Eissa *et al.* (1987) investigated a control group of 15 women who were considered as normal fertile because they were treated with donor insemination, having partners with azoospermia (n = 14) and one with a very poor sperm quality. They found 9 out of 39 cycles (23%) abnormal, of which seven could be classified as LUF. The two other cycles showed shrinkage of the follicle before the LH surge, without rupture. Other authors, however, found no abnormal cycles in their control groups (Table 7).

**Table 5.** Incidence of cycle disturbances in patients with regular cycles, as diagnosed by ultrasound in spontaneous cycles in which luteinization of the follicle occurred

| Author | Diagnostic group | No. of cycles and patients | Cycle disturbances |
|---|---|---|---|
| Coutts *et al.*, 1982 | Unexplained subfertility | 27 cycles in 21 patients | LUF cycles in 12 of 27 cycles (44% of cycles) in 8 out of 21 patients (38% of patients) |
| Polan *et al.*, 1982 | Tubal pathology | 14 cycles in14 patients | 5 abnormal cycles (36%): In 3 cycles small follicles that luteinized (21%); in 2 cycles decreasing folllicle diameter, 48 h before the LH surge, without ovulation (14%) |
| Kerin *et al.*, 1983 | Tubal pathology | 85 cycles in 31 patients | LUF in 4 cycles (4.7% of thecycles; 13% of the women) |
| | Endometriosis | 12 cycles in 5 patients | All cycles normal |
| | Unexplained subfertility | 15 cycles in 6 patients | LUF in 1 cycle |
| Daly *et al.*, 1985 | Unexplained subfertility | 33 cycles in 33 patients | 6 abnormal cycles (18%): LUF in 3 cycles (9%); small follicles which ovulated in 3 cycles (9%) |
| Lewinthal *et al.*, 1986 | Unexplained subfertility | 6 cycles in 6 patients | 3 abnormal cycles (50%): LUF cycle in 1 woman; poor follicular growth or premature luteinization in 2 women (follicles 13 and 12 mm) |

**Table 5, Continued.**

| | | | |
|---|---|---|---|
| Eissa *et al.*, 1987 | Unexplained subfertility; minimal endometriosis and/or some evidence of tubal disease | Total group: 89 cycles in 45 patients | Abnormalities in 48 cycles (54% of the cycles): 19 'LUF cycles' (shrinkage of follicles after the LH surge) (21% of the cycles); 21 'cyst cycles' (growing follicles after LH surge) (24% of the cycles); 8 'asynchronous cycles' (shrinkage of follicles 24 h before the LH surge) (9% of the cycles) |
| | | Unexplained subfertility: 47 cycles in 22 patients | Abnormal cycles in 26 of 47 cycles (55%). |
| Petsos *et al.*, 1987 | Unexplained subfertility | 115 cycles in 100 patients | 32 abnormal cycles (28%): LUF in 16 cycles (14% of the cycles); shrinkage of follicles, 4 days before LH surge in 12 cycles(10%); large cysts in the follicular phase, with subsequent luteinization in 4 cycles (3%) |
| Hamilton *et al.*, 1990 | Unexplained subfertility | 175 cycles in 175 patients | 18 LUF cycles (10%) |
| Rodin *et al.*, 1994 | Unexplained subfertility | 35 cycles in 35 patients | 16 abnormal cycles (46%): LUF in 14 cycles (40%); poor follicle growth (<16 mm) or too early LH surge in 2 cycles (6%) |
| Leach *et al.*, 1997 | Unexplained subfertility | 12 cycles in 12 patients | All cycles were normal and ovulatory |

**Table 6.** Recurrence of subtle cycle disorders in patients, as diagnosed by ultrasound

| Author | Patients with subtle cycle disorders who were examined in more than 1 cycle | Cycle characteristics |
|---|---|---|
| Coutts *et al.*, 1982 | 8 patients with spontaneous LUF cycles | In total 12 LUF cycles in these 8 patients, which means recurrence in some of them |
| Polan *et al.*, 1982 | 5 patients with an abnormal spontaneous cycle | In a second cycle 1 cycle again was abnormal |
| Kerin *et al.*, 1983 | 8 patients with a spontaneous LUF cycle | Recurrence of LUF in 1 out of 35 repeated cycles |
| Hamilton *et al.*, 1985 | 27 patients with a LUF cycle in a spontaneous or stimulated cycle | Recurrence in 13 of 45 repeated cycles; recurrence ocurred in 6 patients |
| Daly *et al.*, 1985 | 3 patients with a spontaneous LUF cycle | In a second cycle 2 of the 3 patients had again a LUF cycle |
| Lewinthal *et al.*, 1986 | 2 patients with spontaneous cycles with 'poor follicle growth'; one patient with a LUF cycle | One patient in two successive cycles again 'poor follicle growth'; the patient with a LUF cycle had again a LUF cycle during 3 consecutive cycles |
| Eissa *et al.*, 1987 | 45 patients with 2 or more cycles | 22 normal first cycles were followed by 11 normal and 11 abnormal cycles;<br>23 abnormal first cycles were followed by 14 abnormal and 9 normal cycles |

**Table 7.** Cycle characteristics in control groups, as investigated by ultrasound

| Author | No. of cycles and women | Cycle disturbances or characteristics by ultrasound |
|---|---|---|
| Geisthövel *et al.*, 1983 | 6 cycles in 5 volunteers, 22-30 years of age | All cycles were ovulatory |
| Zegers-Hochschild *et al.*, 1984 | 28 cycles in 25 multiparous volunteers, 21-30 years | 13 conception cycles, 15 non-conception cycles. All cycles were ovulatory |
| Eissa *et al.*, 1987 | 39 cycles in 15 women, treated with donor insemination, 23-40 years (median 31) | 9 abnormal cycles (23%): 7 LUF cycles (18%); 2 'asynchronous cycles' |
| Hamilton *et al.*, 1990 | 43 cycles in 43 volunteers, 18-36 years (median 26); at least half of them have conceived since | All cycles were ovulatory |
| Bakos *et al.*, 1994 | 16 cycles in 16 volunteers; most of them had been pregnant | All cycles were ovulatory |
| Leach *et al.*, 1997 | 12 cycles in 12 volunteers with proven fertility, 25-40 years (mean 32.8) | All cycles were ovulatory |

**Table 8**. Treatment of LUF and other subtle cycle disorders in subfertile women with regular cycles

| Author | Cycle disorder | No. of patients | Treatment | No. of patients with ovulatory cycles | No. of patients pregnant |
|---|---|---|---|---|---|
| Daly, 1989 | Recurrent LUF in 3 cycles | 9 | Clomiphene citrate or hMG/hCG | 9 | 8, after 1-6 ovulatory cycles (mean 2.4) |
| | LUF, rupture of small follicles, abnormal follicle growth, in 2 or more cycles | 16 | Clomiphene citrate, hMG/hCG or dexamethasone (1 patient) | 13 | 11, after 1-5 ovulatory cycles (mean 2.6) |
| Check *et al*., 1992 | LUF, premature luteinization, in 2 consecutive cycles | 46 | Clomiphene citrate, hCG, hMG/hCH; in total 198 cycles | 45 | 24 (12.1%/cycle) |
| Rodin *et al*., 1994 | LUF in a first cycle | 14 | One clomiphene citrate-stimulated cycle | 11 | |

## Subtle cycle disorders in unexplained subfertility

Special attention has been paid to the cycles of patients diagnosed as having 'unexplained subfertility'. In these patients, even after an extensive diagnostic work-up, no cause of the infertility can be identified. It seems rational to search for subtle cycle disorders in these women. The prevalence of unexplained subfertility in the literature ranges from 8% to 37% (Leach *et al.*, 1997). Differences not only depend on the levels of investigation, but also on characteristics of the patients groups and referral patterns. Publications on disorders of follicle growth and ovulation in unexplained infertility are included in Table 5. The incidences has been reported to range from 0% (Leach *et al.*, 1997) to as much as 54% (Eissa *et al.*, 1987) and do not differ from those in the total group of subfertile women. In Table 5 within the unexplained group, 465 cycles are summarized, in which 112 subtle cycle disorders (24%) were observed.

Summarizing, subtle cycle disorders, especially LUF, rarely occur in normal, fertile women, in contrast to subfertile patients in which they occur much more often. It is reasonable to assume that such cycle disturbances explain at least partly, the subfertility in such couples. A comparison can be made with oligomenorrhoeic patients. Although most of them will ovulate sometimes, the number of ovulatory cycles is less than normal. There is general agreement that these patients benefit from ovulation induction treatment. Patients with one or more LUF cycles may have a diminished probability to conceive, although this has not been proven in large longitudinal studies. It is our impression that some women may benefit from diagnosing subtle cycle disorders.

The etiology of subtle cycle disorders has not been established. In rats, LUF cycles can be induced by manipulating the LH surge (Plas-Roser *et al.*, 1985; Mattheij *et al.*, 1995), and local (ovarian) disorders of prostaglandin synthesis may play a role (Plas-Roser *et al.*, 1985). In women, a central cause has been suggested because a blunted LH surge has been found in LUF cycles (Hamilton *et al.*,1985; 1987). However, arguments for a peripheral (ovarian) cause are suggested by the same group (Hamilton *et al.*, 1985) because they ob-

served recurrence of LUF especially in patients with a history of PID. Recurrent LUF cycles during ovulation induction could have an extra-ovarian as well as an ovarian origin.

Treatment, with the intention to reverse the typical abnormality that has been found in a patient (for instance enhancing the LH surge in case of a LUF cycle, with hCG), seems not indicated, because in different cycles different disturbances may occur (Eissa *et al.*, 1987). Some authors report on treatment of patients in whom LUF cycles were found by use of ultrasound, with regimens that are used in patients with oligo- or amenorrhoea, who wish to conceive. Results are summarized in Table 8. More than half of the patients became pregnant. Although these treatments seem reasonably successful, it remains unknown whether the reported pregnancies are treatment-dependent, or treatment-independent. Randomized studies comparing treatment cycles with untreated controls have not been published.

## Subtle cycle disorders during ovulation induction treatment

Few studies have addressed cycle characteristics and cycle disorders during ovulation induction in oligo- or amenorrhoeic patients with clomiphene citrate, gonadotropins or pulsatile GnRH. The occurrence of multifollicular growth and the resulting multiple gestations are known from the initial use of these drugs. In Table 9, data on treatment of cycle disorders that are subtle, are summarized. These preliminary data indicate that LUF cycles may occur in a significant part of the cycles. Check *et al.* (1990) noted that almost all patients with a LUF in their first cycle showed recurrence in the second one. In cycles that are stimulated with HMG, ovulation rates depend on follicle diameter. Silverberg *et al.* (1991) followed the destiny of all follicles in controlled hyperstimulation cycles before IUI. Follicles = 17 mm on the day of hCG-administration had a 13-fold incidence of ovulation compared to follicles = 16 mm. Follicles = 14 mm rarely ovulated. Clomiphene citrate is also used to induce controlled hyperstimulation in patients with regular cycles, in combination with IUI. A remarkable observation was made by Randall and Templeton

(1991) who stimulated 24 women with unexplained subfertility and sonographically proven ovulatory cycles. In cycles stimulated with CC, six of these women had a LUF cycle.

**Table 9.** Cycle characteristics of ovulation induction cycles in women with oligomenorrhoea or amenorrhoea

| Author | No. of cycles and women | Cycle disturbances or characteristics by ultrasound |
|---|---|---|
| Hamilton *et al.*, 1987 | 45 cycles stimulated with clomiphene citrate | 6 LUF cycles (13%) |
| | 14 gonadotropin-stimulated cycles | No LUF cycles |
| | 10 GnRH-stimulated cycles | 4 LUf cycles (40%) |
| Check *et al.*, 1990 | First cycles in 220 patients, stimulated with HMG | 16 LUF cycles (7%) |
| | Second cycles in 197 patients | 13 LUF cycles (7%) |

## The luteal phase defect

The luteal phase of the normal menstrual cycle starts at the time of ovulation and ends at the onset of menstruation. Under the influence of progesterone produced by the corpus luteum secretory changes are induced in the endometrium, which has been proliferated under the influence of estrogens. A secretory endometrium is a prerequisite for implantation of the early embryo and for maintenance of pregnancy (Csapo and Pulkkinen, 1978). It has been assumed that the luteal phase may be abnormal, a so-called luteal phase defect (LPD), being a cause of infertility (Jones, 1976). LPD is also known as luteal phase deficiency (Balasch *et al.*, 1986), luteal phase inadequacy (Murthy *et al.*, 1970) or luteal phase insufficiency (Taubert, 1978). LPD is a controversial issue, much so because of a lack of consensus in its diagnostic criteria (Li and Cooke, 1991; Castelbaum and Lessey, 1995). In our opinion, a LPD-diagnosis should only be suggested in an *ovulatory* cycle. For evaluating LPD several methods have been used, including basal body temperature, sonographic measurement of endometrial thickness, histological dating of the endometrium (Noyes *et al.*, 1950) and measurements of progesterone in plasma (Hull *et al.*, 1982) or saliva (Walker *et al.*, 1981). Timing and interpretation of histological dating and of (one or repeated) progesterone assays are a matter of ongoing debate (Li and Cooke, 1991; Castelbaum and Lessey, 1995). Whether LPD really exists has not yet been esthablished. However, in some studies (Li *et al.*, 1991), in infertile populations a significantly higher prevalence (diagnosed by dating of endometrial biopsies) has been found than in fertile control populations. A follow-up study suggests that women in whom LPD has been diagnosed, have reduced fertility compared to women without LPD (Klentzeris *et al.*, 1990). It has been suggested that LPD could be the consequence of an aberrant follicular phase, in which inappropriate estrogen production results in inadequate priming of the endometrium (DiZerega and Hodgen, 1981). However, this could not be confirmed by others (Li *et al.*, 1991). Subnormal progesterone production by the corpus luteum, is another possibility, though unlikely, as Li *et al.* (1991) found that in a majority of cases with retarded endometrial development progesterone levels were normal. Therefore, an

abnormal response of the endometrium to progesterone has been considered the most likely cause of LPD (Li and Cooke, 1991). Many methods of treatment for LPD have been used, including the administration of progesterone, clomiphene citrate, FSH, bromocriptine and pulsatile GnRH. None of these treatments is based on a properly conducted clinical trial including a sufficient number of patients and controls (Karamardian and Grimes, 1992). The only controlled trial on the use of progesterone (Balasch *et al.*, 1982) included 44 infertile patients with histologically documented inadequate luteal phase who were randomly divided into 3 groups. Sixteen patients received progesterone as vaginal suppositories, 16 dehydrogesterone orally, and 12 no treatment. Both treatment regimens corrected the inadequate luteal phase compared to the controls ($P < 0.001$). Pregnancy rates showed a trend in favour of treatment, but the differences were not statistically significant (five pregnancies in the progesterone-group, as well as in the dehydrogesterone group; two in the control group; $P = 0.66$). To be able to show a difference, many more patients would have been required (Karamardian and Grimes, 1992). The most rational treatment should be aimed at improving the responsiveness of the endometrium to progesterone (Li and Cooke, 1991). Women above age 40 may have diminished uterine receptivity for nidation of embryos. In oocyte donation using oocytes of young donors, implantation rates could be enhanced by applying supraphysiological progesterone replacement (Meldrum, 1993). Preliminary results suggest that low-dose aspirin may improve implantation rates in oocyte donation recipients with thin endometrium (Wada 1994, Weckstein 1997).

## Concluding remarks

Much is known of the anatomy and normal function of the organs that govern the menstrual cycle. Hormonal and other signals, induction of hormone receptors, and feedback systems that regulate follicle growth and ovulation have been studied extensively. However, it seems almost impossible to describe the hormonal and ultrasonic conditions a cycle must fulfill to be considered as normal. Hormonal levels as well as follicle growth vary greatly be-

tween women, and in the same women, between cycles. In subfertile women with regular cycles more often cycle disorders have been found than in controls. Cycles can be monitored by determination of several hormones throughout the cycle, and by ultrasound scans of the ovaries. It is still not clear how often cycle abnormalities occur in a general subfertile population, and how often they contribute to the subfertility of these women. The costs which are necessary for diagnosing such disorders have not been calculated so far. Moreover, the benefits of treatment of subtle cycle disorders are unknown. In women with oligo- or amenorrhoea, ovulation induction is an established treatment, but how often ovulation disorders are induced by the treatment, is not clear. In patients who are treated with CC, pregnancy rates are less than expected from the supposed ovulation rates (Hammond *et al.*, 1983, 1984). The cause of this discrepancy is still unknown.

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# Chapter 2

INTRODUCTION, PART 2

HORMONES AND REPRODUCTIVE AGING (with an abstract)

P. van Zonneveld, G.J. Scheffer, F.J.M Broekmans, E.R. te Velde

Department of Reproductive Medicine, Division of Obstetrics, Neonatology and Gynecology, University Medical Center Utrecht, Heidelberglaan 100, 3584 CX Utrecht, The Netherlands

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**Abstract**

Female fertility decreases with age. Postponement of childbearing, which is a remarkable demographic trend in Western countries, therefore leads to an increasing proportion of subfertile couples. The stock of primordial follicles is established during early fetal life and decreases exponentially thereafter by a process of follicle atresia. According to the so-called ovarian hypothesis, a decrease in both quantity and quality of the remaining resting follicle pool dictates several reproductive events including the beginning of subfertility, the end of fertility, the age of transition from cycle regularity to cycle irregularity, and the age at menopause. Age at menopause is the only clearly recognizable reproductive event and marks the exhaustion of follicular reserve. The same variation of age at menopause is probably also present for the preceding reproductive events. Why individual follicles start to develop, is unknown. Follicle stimulating hormone (FSH), which is necessary for growth of the selectable follicles, and maturation of the dominant follicle, may play an additional role. The same factors (mainly genetic) that determine age at menopause may also determine reproductive aging. Though a decrease of fertility starts relatively early in the reproductive life of a woman, hormonal changes as well as ovulation disorders and irregular cycles occur much later. The first endocrine sign of reproductive aging is a monotropic rise of early follicular FSH levels, which becomes clear after age 40, with a large overlap with levels in young women. The patterns of luteinizing hormone (LH), estradiol ($E_2$) and progesterone do not change with age, as do follicle growth patterns. Inhibin B shows a higher rise after the LH peak in younger women, The relevance of this rise is not clear. Until the primordial follicle pool is almost exhausted, hormonal parameters do not seem to be reliable markers of reproductive aging.

## 1. Introduction

Delaying childbearing is a remarkable demographic trend in all Western countries [1,2], which started in the early 1970s when oral contraceptives became

widely available. From that time onwards, having children could be planned and, as a result, postponed in order to complete an education or give preference to a career. Postponement of childbearing, however, inevitably leads to attempts to become pregnant at a more advanced age. This trend considerably contributes to the increasing proportion of subfertile couples [2]. Van Noord-Zaadstra et al [3] found a rapid decline of the monthly chance to have a pregnancy resulting in the birth of a healthy child, after the age of 30 years in couples who tried to conceive with the aid of donor-sperm insemination. At 35 years, this monthly chance was halved compared to the period before age 30, and at 38 years, it had dropped to one-quarter. A further decrease of fecundity is illustrated by the mean age at the birth of the last child (40-41 years), found in natural populations when no contraception was used [4,5]. Other reflections of diminishing fecundity after age 37-38 years are a lower implantation rate per embryo in in-vitro fertilisation (IVF) [6], as well as a lower probability of a pregnancy after IVF [7]. Age-related declining fecundity is a result not only of a decreasing monthly fertility rate, but also of an increasing abortion rate, rising from about 10% before age 30 to 45% at age 45 [8].
At the end of female fertility, most women still have regular menstrual cycles with progesterone production in the luteal phase, as suggested by a biphasic basal body temperature. Only at about the age of 45 years do menstrual cycles become irregular, some 6 years before menopausal age [9], which comes at a mean age of 50-51 years in Western countries.

## 2. Concepts of reproductive aging

The stock of primordial follicles (oocytes surrounded by granulosa cells) in the ovaries, established during fetal life, has to serve the reproductive needs of a woman for the rest of her life. At about 20 weeks of gestation, a maximum number of about 7 million has been reached [10-13]. Only a few hundreds will eventually mature to the stage of dominance, and ovulate. All the others -the vast majority- will become atretic by apoptosis [11,14]. This process of atresia starts already before birth. At birth and at menarche, about 1 000 000 and 300 000 are left, respectively. At a mean age of 37-38 years, only about 25 000

resting follicles are present in the ovaries. Thereafter, the curve follows a biphasic pattern because of an acceleration of the disappearance of follicles [11]. Menstrual cycles become irregular at about age 45 [9], which is on average 6 years before menopause. Cycle irregularity appears to be dependent on the number of remaining follicles and not on age [12]. This is supported by the finding that the time interval between the beginning of cycle irregularity and age at menopause is 6-7 years, regardless of the age at menopause [15]. When almost no follicles are left, menopause occurs.

According to the so-called ovarian hypothesis, decreasing fertility after age 30 is dictated by the decreasing quantity [11] and quality [16-18] of the resting follicle pool. Whether the differentiation of oocyte quality is already established during fetal life or whether quality deteriorates by accumulation of damage thereafter is a matter of scientific debate [19]. The remarkably good result of IVF in (pre)menopausal women when using oocytes of young donors further emphasizes that the oocyte rather than the endometrium dictates the age-dependent fertility loss [20]. Although the ovarian hypothesis is widely supported, an additional role of the hypothalamus cannot be ruled out. The so-called neuro-endocrine hypothesis of reproductive aging considers aging of the hypothalamus as the main initiator of a cascade of events leading to menopause. Reproductive aging is considered as a dysregulation of the GnRH pulse generator by a progressive lack of neuro-chemical control from other brain centers. [21]. One of the first signs of this transition is the monotropic rise of FSH during the early follicular phase, which causes the acceleration of follicle depletion. In a recent study by Matt et al. [22], changes in LH-pulse patterns were demonstrated in regularly cycling women of 39 or older as compared to a younger age group. These alterations may well be the first sign of hypothalamic aging.

In Fig. 1, the decreasing resting follicle pool as schematically depicted from the work by Faddy et al. [11] and the corresponding reproductive events are summarized. This figure suggests that the decreasing resting follicle pool is closely related to age-related reproductive events including age at menopause.

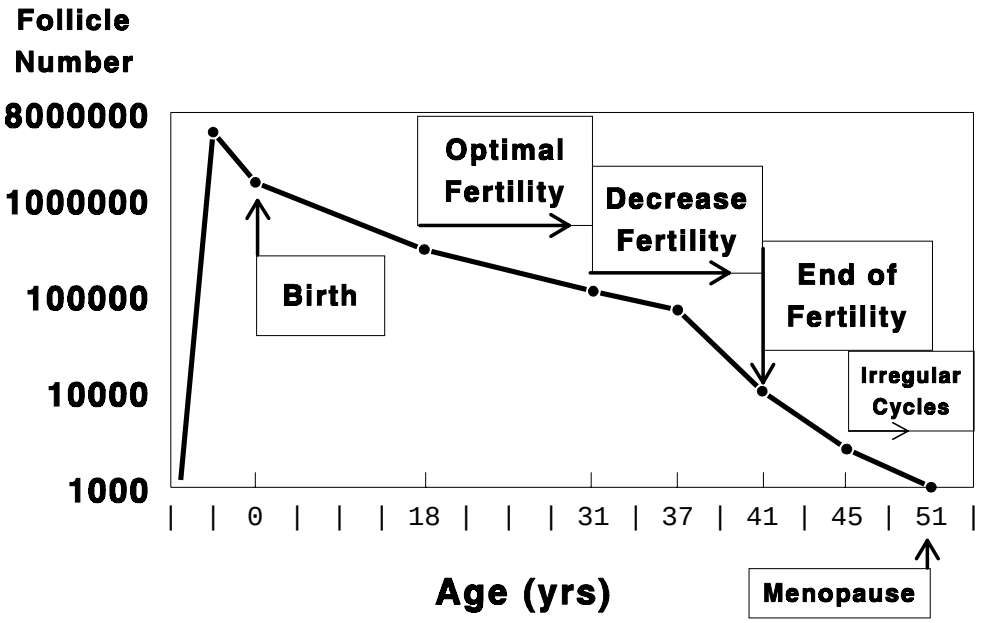


Fig. 1. Decreasing follicle pool and the corresponding reproductive events.

The dynamics of follicle growth from the resting or primordial stage until ovulation is shown in Fig.2 and is based on the work of Gougeon [13]. Why, after a state of quiescence, which may last several decades, individual follicles start to grow, remains a mystery. The entire process from the initiation of growth until ovulation takes at least half a year [13]. However, the vast majority of follicles will become atretic by apoptosis at some stage of development before the final stage of maturation for ovulation has been reached. For example, antral follicles measuring 2-10 mm will become atretic, unless rescued by the intercycle FSH rise. From this cohort of so-called selectable foIlicles, which can be visualized by sonography during the early follicular phase, the dominant follicle destined to ovulate will be selected during the first 10 days of the menstrual cycle. The continuous initiation of growth of resting follicles results in an increasing depletion and, ultimately, exhaustion, of the entire resting follicle pool. Whether resting follicles can go directly into atresia before initiation of early growth, is still unknown.

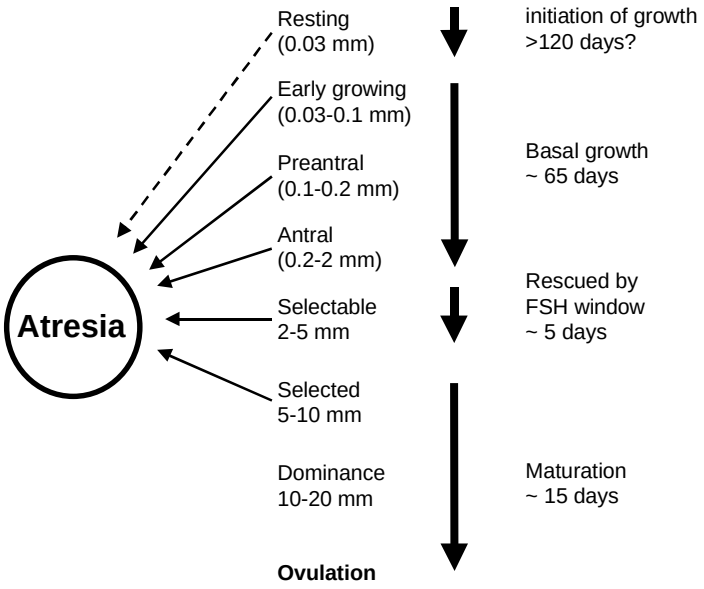


Fig. 2. Various follicle stages in relation to the follicle diameter and the phase and time of growth in women with normal, menstrual cycles.

Development of the selectable follicles and the maturation of the dominant follicle is only possible in the presence of FSH. Which factors determine the initiation of growth of the resting follicles is unknown, although FSH may play an additional role. It is certain that FSH is no sine qua non because selectable follicles are present in circumstances in which FSH levels are very low [23,24] or absent, as in Kallmann patients [25,26]. There is, however, some evidence for a facilitating role of FSH in initiating follicular growth or stimulating early follicular growth. For example, Zheng et al. [27] showed that FSH mRNA is expressed in human resting follicles, which suggests a possible influence of FSH. Very low or absent FSH levels were found to inhibit follicular depletion in animals [28,29] and to inhibit cyclophosphamide-induced follicular depletion in monkeys [30]. A stimulating influence of FSH is also suggested by the accelerated depletion of the resting follicle pool once FSH levels start to rise [11].

Using a mathematical model, Faddy and Gosden [31] provided estimates of the daily number of follicles entering the growth phase. They found a dramatic drop of 45 follicles in the age group 20-30 years to six follicles in age group 38-45 years. The question of how many of these will reach the selectable stage is addressed by Scheffer et al. [32]. They found that the mean number of sonographically detectable follicles with a diameter of at least 2 mm in the early follicular phase decreased less steeply. This may indicate that the fraction of follicles at the selectable stage increases as the size of the total follicle pool decreases: at an older age less follicles enter the growth phase, but relatively more of them survive. Data from other research groups confirm this concept [33-35]. Scheffer et al. [32] also found a high correlation between the number of selectable follicles and calender age in 140 regularly cycling volunteers with proven fertility. Whether assessments of the number of antral follicles can be used as a measure of reproductive age in women with subfertility, or as a prognosticator of the ovarian response to controlled ovarian hyperstimulation, deserves further study.

## 3. Age at menopause as a key event of reproductive aging

All ages mentioned so far, and presented in Fig. 1, are given as mean or median values. Menopause, for example, comes at a mean age of 51 in most Western countries, but shows an enormous variation. It is the only clearly recognizable reproductive event and marks the definite exhaustion of follicular reserve. We hypothesize that the sequence of preceding reproductive events - the beginning of subfertility, the beginning of infertility, and the transition from cycle regularity to irregularity- is closely related to age at menopause and shows a similar age variation. The cumulative distribution of age at menopause is depicted by the curve at the right in Fig. 3 and is based on data by Treloar [9].

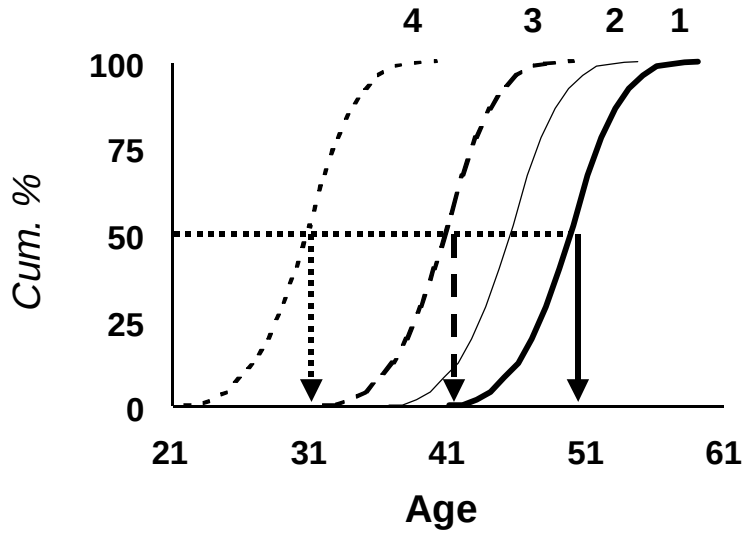


Fig. 3. Cumulative age at menopause (1), age of transition from cycle regularity to irregularity (2), the end of fertility (3) and the beginning of subfertility (4).

Transition of cycle regularity to irregularity has been shown to occur at a mean age of 45-46. The mean age of last delivery in natural populations, which is likely to reflect the end of female fertility, has been found to occur at age 40-41 [4,5] and the beginning of subfertility at about 31 years of age [3] or even earlier [5]. If, indeed, these reproductive events have a similar age variation to menopause, cumulative age at menopause, age at transition from cycle regularity to irregularity, end of female fertility and beginning of subfertility can be presented as shown in Fig. 3 by curves 2, 3, and 4, respectively. This would mean that a woman who will have her menopause, for example, at age 45, starts to be subfertile at age 26 and is already infertile at age 36 while her cycles are still regular and seemingly normal. Delay of childbearing can impose great problems in such women.

If age at menopause is a reliable marker of reproductive aging, the same

factors that determine age at menopause also determine reproductive aging. Several factors are known to influence age at menopause. They can be divided in lifestyle factors, factors related to fecundity and possibly events during intrauterine life. For a review of this subject, see Ref. [36].
In spite of the fact that most of these factors show a highly significant relation with age at menopause, multivariate analysis reveals that only 1-3% of the variance of age at menopause is explained by lifestyle and fecundity-related factors [37]. Genetic factors seem a much more likely explanation. A correlation is apparent between age at menopause of mothers and daughters [38] and also between sisters with premature ovarian failure [39]. In a study of 361 sisters and 5881 women from an at-random population, we demonstrated that more than 80% of the observed differences in the age of menopause have a genetic origin commonly referred to as heritability (J.P. de Bruin et al., submitted for publication). Two studies on menopausal ages in identical twins [40,41] come to similar conclusions. If, indeed, the age at menopause and the preceding reproductive events are closely related, it is most likely that they are determined by the same genetic factors.

## 4. Endocrine changes and follicular development related to aging

In the following section, we give an overview of age-related changes in the gonadotropic hormones [follicle stimulating hormone (FSH) and luteinizing hormone (LH)], the main hormones secreted by the ovaries during the hormonal cycle [estradiol ($E_2$), and progesterone] and the peptide hormone inhibin. Some details of follicular development in relation to hormonal changes are briefly discussed.

### *4.1. Follicle stimulating hormone (FSH)*

The first endocrine sign of reproductive aging is a monotropic rise of early follicular FSH levels in regularly cycling women between age 35 and 40 [42-47]. Schipper et al. [48] did not observe any age-related difference in early follicular FSH in 38 strictly regularly cycling volunteers, but this may be explained by the mainly young age of these volunteers. Ahmed Ebbiari et al. [49] described an early follicular FSH rise from age 27 years on, in a large group of patients

with a fertility problem. In this patient group, however, a selection of women with (still hidden) ovarian failure may have been included. In 165 proven fertile volunteers between the ages of 25 and 47 years, we also found a rise of early follicular FSH related to age ($R$= 0.25, $P$ = 0.001) (Fig. 4).

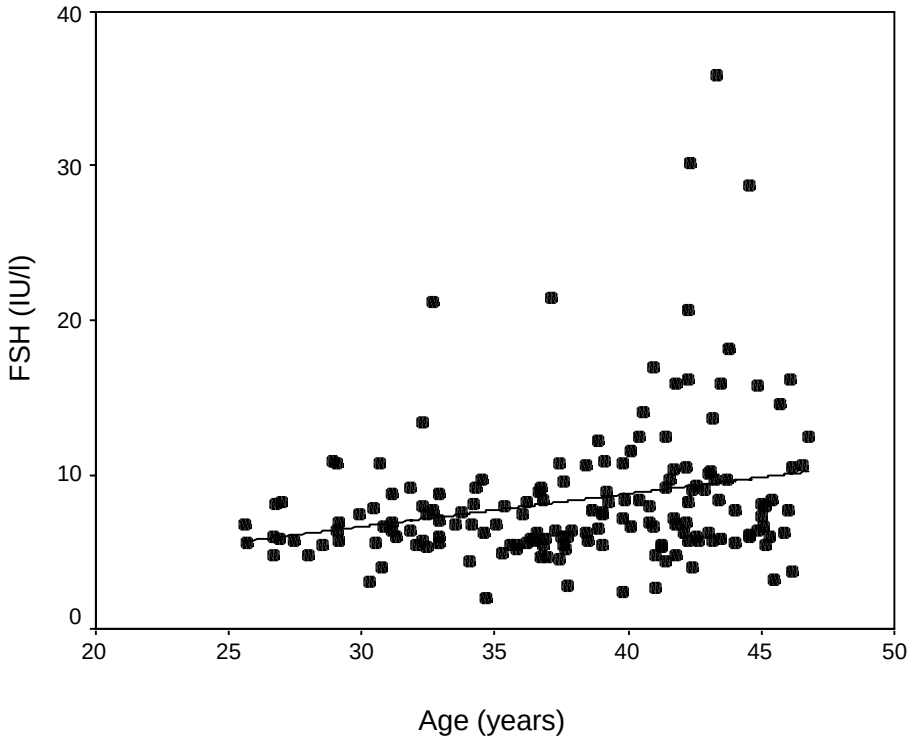


Fig. 4. Early follicular phase FSH levels in 165 volunteers with regular menstrual cycles. ($R$ = 0.25, $P$ = 0.001).

However, this correlation was strongly influenced by the elderly women, because below age 40, the correlation was much weaker and did not reach statistical significance ($R$ = 0.15, $P$ = 0.125). Moreover, FSH levels in older women show a large overlap with levels in young women. This observation is in line with the threshold concept of Brown [50], which suggests that women have an individual FSH level above which follicles are stimulated for further growth. This means that a moderately elevated early follicular FSH either may reflect a

high but physiological threshold level or may be a sign of early ovarian failure. The FSH rise is also seen in other phases of the cycle [42,43,45,47]. Our data from 81 proven fertile volunteers of whom we followed a cycle by ultrasound measurements of follicle development as well as by hormone assays (Fig. 5) show a higher mean FSH level in the older group during the follicular phase including the day of the LH peak, although the differences do not reach statistical difference. The above-mentioned studies are all transversal studies. Subtle longitudinal FSH changes within the normal range may well occur much earlier.

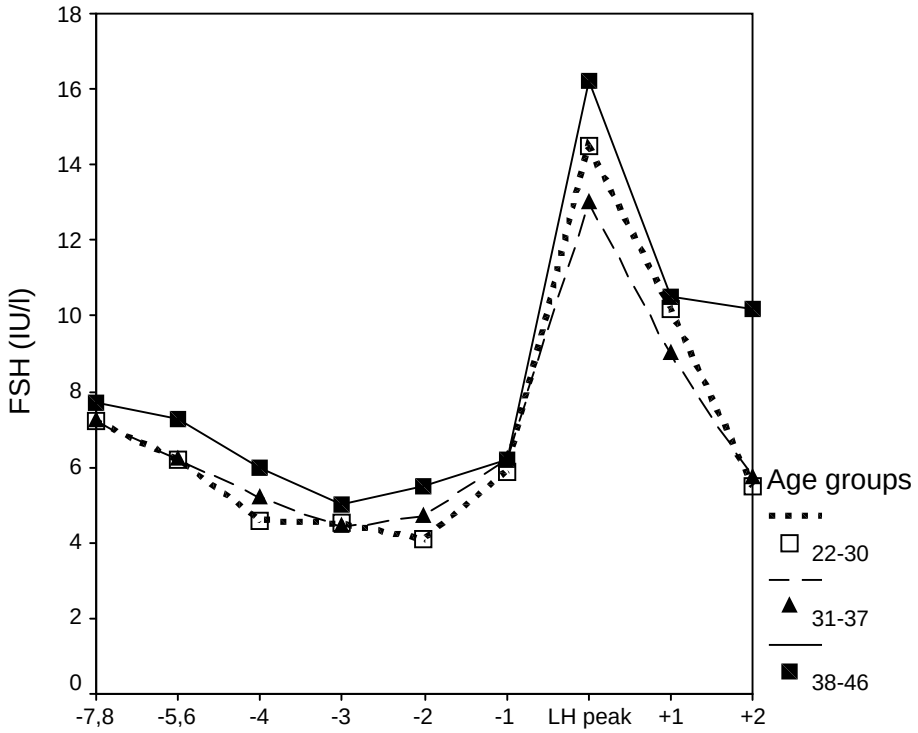


Fig. 5. Mean FSH levels in three age groups of 81 volunteers, in relation to the day of the LH peak.

*4.2. Luteinizing hormone (LH)*

In normal volunteers, several research groups were able to confirm an LH rise with age [42-46], except Klein et al. [47], who found no difference. In our group of volunteers we found equal LH levels in the early follicular, late follicular and luteal phase. The LH peak levels during the LH surge were slightly higher in the youngest group compared to the two other groups, however, without a statistically significant difference (Fig. 6). In a patient group, Ahmed Ebbiari et al. [49] described an early follicular rise of LH with age, but 5-10 years later than the age-related FSH rise.

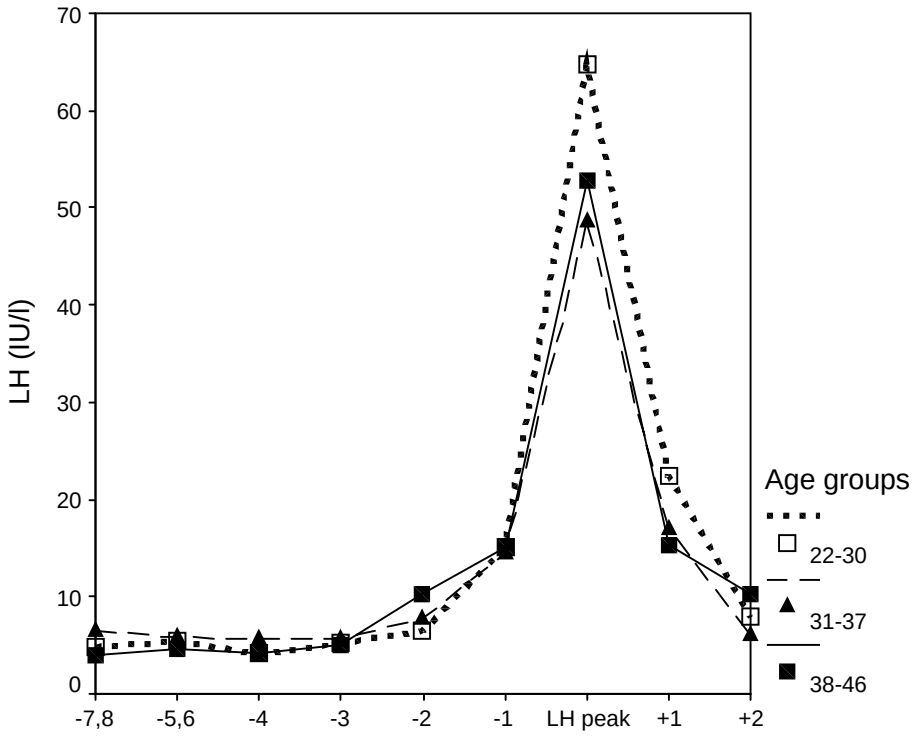


Fig. 6. Mean LH levels in three age groups of 81 volunteers, in relation to the day of the LH peak.

### *4.3. Estradiol ($E_2$)*

Inconsistent patterns have been reported on early follicular estradiol levels at older ages. Sherman et al. [42] found a decrease, Reyes et al. [43] no change, whereas in contrast, Musey et al. [44] and Klein et al. [47] found early follicular $E_2$ levels that were increased in older, compared to younger women. Fig. 7 shows the mean $E_2$ concentrations plotted against the day of the LH peak level in our group of volunteers. No differences are observed between the three age groups.

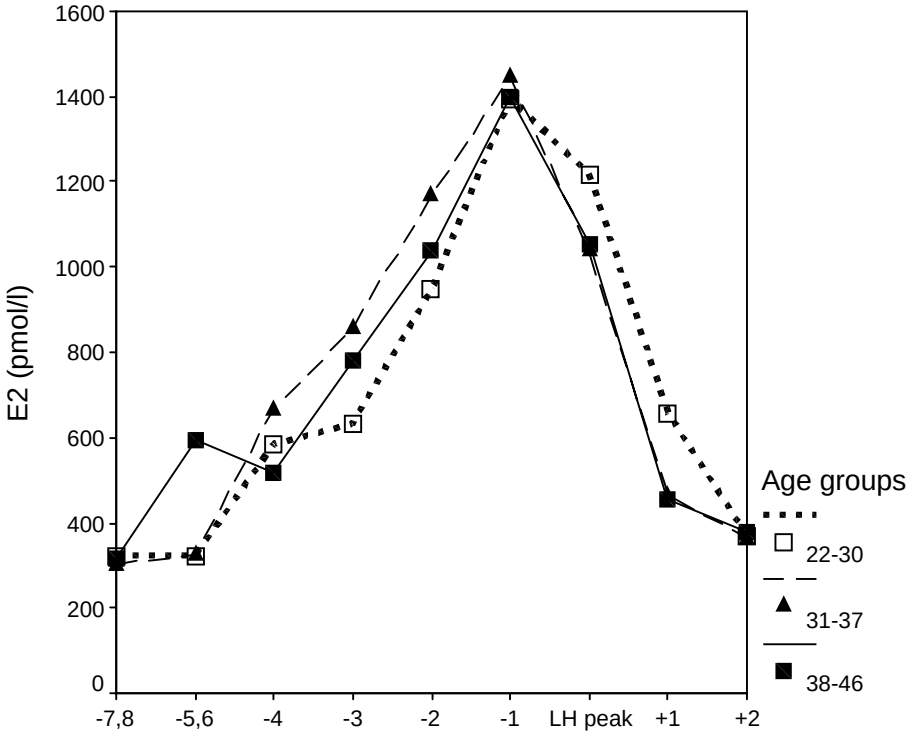


Fig. 7. Mean estradiol levels in three age groups of 81 volunteers, in relation to the day of the LH peak.

### 4.4. Progesterone

Progesterone levels do not appear to show any clear changes with increasing age. As well as a decrease [43], no change [42,46] as a tendency to increase with age [47] have been described. The data of our own group have not yet been analyzed.

### 4.5. Inhibin B

Inhibin is a heterodimer secreted by granulosacells, which is able to selectively inhibit FSH secretion. Circulating forms of inhibin include inhibin A (composed of a and ßA subunits) and inhibin B (composed of a and ßB subunits). During the normal menstrual cycle, a significant inverse correlation is present between early follicular FSH and inhibin B [51]. Inhibin A rises parallel to $E_2$, and is thought to reflect dominant follicle function. Thus, inhibin B seems mainly to be related to early follicular growth and therefore may be a marker of the age-dependent decrease of the follicular pool [52]. In our study of regularly cycling proven fertile women, aged 25-47 years, we also found an inverse, but rather weak, correlation between inhibin B on cycle day 3 versus age ($R = 0.12$, $P = 0.14$, $n = 164$). An unexpected observation in our group of volunteer women, as seen during and after the LH surge, is shown in Fig. 8. Comparable levels of Inhibin B were found during the early follicular phase and on the day of the LH peak value, irrespective of age. One day later, however, Inhibin B shows a rise in the younger compared to the older women (22-33 years versus 38-46 years: $p = 0.002$). This suggests an age-related difference in the ability of peri-ovulatory follicles to secrete inhibin B as a response to the midcycle gonadotropin surge. It is not known whether such a different response is of any physiological or clinical relevance.

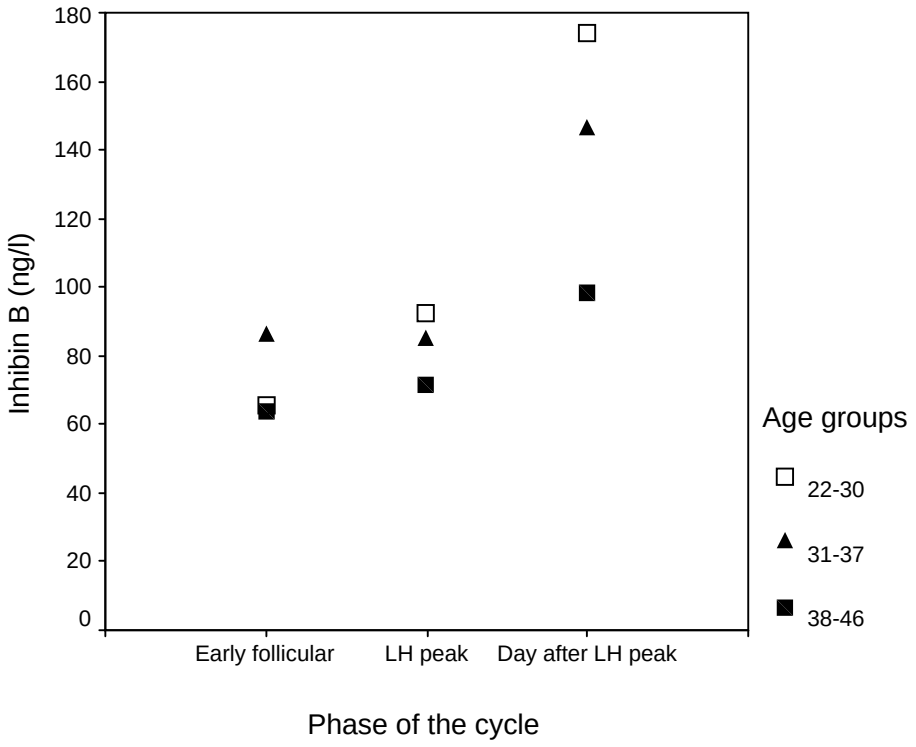


Fig. 8. Mean inhibin B levels in three age groups of 81 volunteers in the early follicular phase, on the day of the LH peak and the day after the LH peak.

### *4.6. Follicle development*

In Fig. 9, the mean diameters of the developing follicles, as calculated from vaginal-ultrasound measurements of three perpendicular dimensions of the leading follicles, are shown. Displayed against the number of days preceding the LH peak, there is no difference between the curves of the three age groups among our volunteers. This suggests that the follicle growth pattern does not change with age. The shorter follicular phase in older women, as found by Treloar [9], can possibly be explained by an earlier start of follicle growth in this age group.

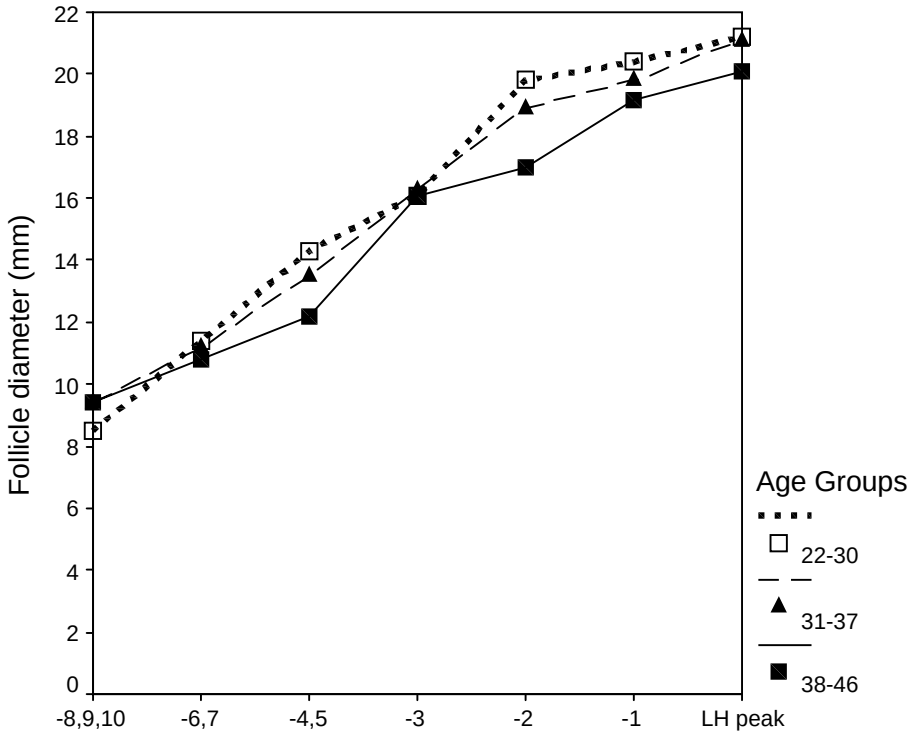


Fig. 9. Mean follicle diameters in three age groups of 81 volunteers, in relation to the day of the LH peak.

## 5. Concluding remarks

It is generally accepted that reproductive aging is directly related to the remains of the stock of primordial follicles, which is established during fetal life (ovarian hypothesis). However, whether aging of the hypothalamus plays an additional role, cannot be ruled out (neuro-endocrine hypothesis). The follicle pool progressively empties as a woman grows older and is (almost) completely exhausted when menopause is reached. Throughout a woman's life until menopause, follicles leave this pool by initiation of growth [13]. Apparently, the decrease in oocyte quantity parallels decrease in oocyte quality, because in addition to a lower probability of conceiving, the incidence of spontaneous

abortions, chromosomal abberations and congenital malformations progressively increases as a woman grows older. Subfertility only starts at a mean age of about 30-31, when the remaining follicle reserve has become a fraction of its original number. Thereafter, a further decrease in both oocyte quantity and quality dictates the subsequent reproductive events: the end of fertility, the beginning of cycle irregularity and, when almost no follicles are left, the occurrence of menopause. The remarkable variation of age at menopause is probably also present for the preceding reproductive events and is mainly genetically determined.

Though a decrease of fertility starts relatively early in the reproductive life of a woman, hormonal changes as well as ovulation disorders are observed much later. From age 41 to 46, a period during which a majority of women are already (or become) infertile, cycles usually are still regular, while not only estradiol and progesterone levels, but also follicular growth and ovulation (as visualized by ultrasound), appear to be normal. Follicular growth including ovulation, and the aforementioned hormone levels, can be considered as a reflection of granulosa cell function, while oocyte quality determines a woman's fertility. Apparently, changes in oocyte quality and granulosa cell function are the results of different, albeit related, processes, the latter being preserved much longer than the former. Only at a median age of about 46 when the primordial follicle pool is almost exhausted [11], do normal cycle characteristics disappear [53]. At this time the size of the antral follicle cohort arriving in the inter-cycle FSH window [54], apparently becomes too small to safeguard the normal development of a dominant follicle. Until that age, hormonal parameters do not seem to be reliable markers of reproductive aging.

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# Chapter 3

AIMS AND OUTLINE OF THE THESIS

## Chapter 3. Aims and outline of the thesis

### Introduction

In Chapter 1, a review was given on the present knowledge of cycle disturbances in regularly cycling subfertile women, and at the end some questions that are still open, were summarized. In Chapter 2, we discussed the current knowledge of the age-related decline of female fertility, and changes of hormonal patterns with age, also in women with still seemingly normal and regular cycles. It is still not clear whether disorders of ovulation, disturbed follicle growth patterns, abnormal hormonal regulation or altered time-interrelations between these events contribute to such a decline.

### Aims of the thesis

The aims of the thesis are:

1. To estimate the incidence of subtle ovulation disorders in women with infertility problems, who have a regular menstrual cycle and a biphasic BBT, and:
   To investigate whether ovulation disorders can be predicted by low luteal phase serum progesterone levels, in subfertile women with regular cycles;

2. To evaluate the costs, necessary to diagnose subtle ovulation disorders in women with a history of regular menstrual cycles;

3. To investigate whether ovulation disorders, like LUF cycles, or abnormal hormonal patterns, occur more often in clomiphene citrate-stimulated seemingly ovulatory cycles compared to cycles of age-matched proven fertile women;

4. To obtain reference values for cycle normality from a group of relatively young, proven fertile women who have regular menstrual cycles, and:

To evaluate whether follicle growth, ovulation, endometrial development and hormonal patterns in proven fertile older women differ from the reference group, and whether differences, if present, may explain, or contribute to, the age-related loss of fertility.

## Outline of the following chapters

In Chapter 4, we investigate whether serum progesterone levels in the luteal phase that remain below a pre-set level during two subsequent cycles, can predict the occurrence of subtle ovulation disorders in a following cycle.
Chapter 5 describes the costs of diagnosing ovulation disorders in subfertile women who have regular cycles, by different methods, and discusses the benefits for the patients of such a diagnostic approach and subsequent treatment.
In Chapter 6, we describe hormonal patterns and follicle development and ovulation in cycles of women with oligo- or amenorrhoea, who are treated with the ovulation-inducing agent clomiphene citrate. The results are compared with those of an age-matched control group of proven fertile women.
In Chapter 7, several ultrasound and hormonal parameters are investigated during the cycles of a group of proven fertile volunteers, who have an age in which fertility can be expected to be greatly reduced. Their cycles are compared with those of a relatively young proven fertile women who served as a reference.
In Chapter 8, the results and conclusions of the thesis are discussed and summarized. Directions for future research are indicated.

# Chapter 4

## LOW LUTEAL PHASE SERUM PROGESTERONE LEVELS IN REGULARLY CYCLING WOMEN ARE PREDICTIVE OF SUBTLE OVULATION DISORDERS

P. van Zonneveld, E.R. te Velde and H.P.F. Koppeschaar*

Department of Reproductive Endocrinology and Fertility and *Department of Endocrinology, University Hospital Utrecht, Utrecht, The Netherlands

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## Abstract

Serial hormonal and ultrasound measurements were performed in a group of 50 infertile women with regular menstrual cycles of normal length, and evidence of luteinization by measurement of biphasic basal body temperature (BBT). The progesterone levels however, remained below a critical threshold of 32 nmol/l (1 nmol/l = 0,315 ng/ml) in two cycles.
In 50 cycles, 25 showed definite abnormalities. In 16 other cycles, ovulation was observed, but relatively low luteal progesterone followed. Although pregnancy in these 16 cycles could be less likely, the real significance of this finding is questionable. The etiology of these 'subtle cycle anomalies' is not clear and may be multifactorial. For this reason, no therapy other than use of ovulation-inducing agents by trial and error is as yet available. Preliminary results indicate that cycle disturbances may persist under ovulation induction, even though progesterone levels are normalized.


## Introduction

Although much is known about hormonal and ultrasonic events in the menstrual cycle, no clear data to judge the borders of 'normality' are available. The question arises as to what the characteristics of a normal cycle are.
Follicular growth has been extensively studied. O'Herlihy and colleagues[1] found a pre-ovulatory range of mean follicular diameter of 17-25 mm, and a good correlation between the calculated volume on the basis of ultrasound, and the volume of aspirated follicular fluid. Other groups found similar pre-ovulatory follicle diameters. Zegers-Hochschild and colleagues[2] observed a 95% interval of 18.3-21.0 mm in 13 conception cycles; Eissa and co-workers[3] a range of 18-25 mm in 12 spontaneous conception cycles. From ovulation-induction studies it is known that follicles of 17 mm or larger are more likely to ovulate after administration of human chorionic gonadotropin (hCG) than are follicles of smaller diameter[4]. Follicle rupture is presumed if the follicle has disappeared or markedly decreased in size[5,6]. Fluid in the pouch of Douglas, being confirmatory evidence, is not always seen. Eissa and co-workers[3] observed a decrease in diameter of at least 89% in nine conception cycles, within 48 h after the luteinizing hormone (LH) peak value. They consider a decrease in mean

follicle diameter of at least 60% as evidence of ovulation[7]. The process of follicle rupture is largely unstudied. Pierson and co-workers[8] followed the evacuation of follicle content ultrasonographically in nine cycles. Although a great variability was seen, after a maximum of 21 minutes all follicular fluid was gone. If no follicle rupture is evident, no pregnancies are reported by Eissa and associates[7] in 60 cycles, and by Hamilton and co-workers[9] in 30 cycles, supporting the reliability of this diagnosis. However, good imaging of the ovaries is a prerequisite, as pregnancies have occurred in cycles showing no follicle at all on ultrasonography. After ovulation, the corpus luteum may appear as a cystic structure, as observed by Geisthövel and associates[10] at 2.6 ± 5 days after disappearence of the follicle, stressing the need for daily observations.

In the literature, very few data are reported with regard to E2 levels. 400 pmol/l at the end of the follicular phase is considered to be the lower limit of normal [5,11].

Concerning progesterone values in the normal cycle, in conception cycles Hull and colleagues[12] found mid-luteal progesterone values of 27 - 53 nmol/l as compared to 3 - 80 nmol/l in non-conception cycles. This suggests not only a minimum level, but also an 'optimal range' for fertility. In their study, no visualization of the ovaries was used. Hamilton and co-workers[9] observed 10 of 14 (71%) luteinized unruptured follicle (LUF) cycles if progesterone did not reach 32 nmol/l, in contrast to no more than 10 of 127 (7,9%) abnormal cycles when progesterone was 32 nmol/l or more.

On the basis of the abovementioned data from the literature, we defined the lower limits of normality using the criteria as shown in Table 1.

Usually, women who menstruate regularly and have a biphasic basal body temperature (BBT) are considered to have ovulatory cycles. However, several kinds of ovulation disturbances have been described in such patients. To detect these ovulation disorders, extensive analysis of at least one cycle would be necessary. As mentioned above, plasma progesterone levels might be used as a marker to identify these patients. The present study was undertaken to investigate if ovulation disorders can be predicted by low luteal phase serum progesterone levels, and to estimate the minimal incidence of ovulation disorders in women with infertility problems who have a regular menstrual cycle and biphasic BBT.

**Table 1** Minimal criteria for normal cycle

| |
|---|
| Estradiol (E2) rise of > 400 pmol/l |
| Mean follicular diameter of 17 mm or more at the luteinizing hormone peak level |
| Ovulation (at least 60% decrease of mean follicular diameter) within 48 h after the luteinizing hormone peak |
| Serum progesterone level of at least 32 nmol/l in the luteal phase |

## Materials and methods

The study was conducted at the Department of Reproductive Endocrinology and Fertility of the University Hospital Utrecht.
As a part of the infertility workup in regular cycling women (24-36 days' duration) the quality of the menstrual cycle was assessed by BBT records and measurements of serum progesterone levels in the second half of the cycle. Starting 16 days before the expected day of menstruation, blood was drawn with intervals of 3 to 4 days until menstruation. In the first sample, prolactin also was assayed. If no progesterone value of 32 nmol/l (1 nmol/l = 0,315 ng/ml) or higher was measured, the procedure was repeated in a following cycle. If again the level of 32 nmol/l was not reached, the patient was scheduled for a so-called 'extended cycle analysis' (ECA) as follows. To exclude occult ovarian failure, we measured serum follicle stimulating hormone (FSH) on cycle day 1, 2, or 3[13]. From day 8 onwards: estradiol, LH and progesterone were measured and vaginal ultrasound examination of the ovaries was performed every other day until the mean diameter (mean of the largest diameter and the diameter perpendicular to the first in one plane) reached 13 mm. Subse-

quently, daily measurements of the same parameters followed, until ovulation was observed. Transvaginal ultrasound examination was performed with the use of an Aloka SSD-620 (Aloka Co., Ltd., Mitaba-Shi, Tokyo, Japan) or Toshiba SSH-140 A (Nasu-Works, Nasu, Japan) real-time sector scanner, employing a 5 mHz transducer, calibrated at a sound velocity of 1510 m. Ovulation was defined as disappearance of the leading follicle or diminishing of its mean diameter by at least 60%[7] . If no ovulation was apparent, daily measurements were continued until at least 72 h after the LH peak value was obtained. The patients were then asked to have blood samples taken for estradiol and progesterone measurement with 2-3-day intervals until menstruation.

Hormone determinations were made as follows. LH was measured with a commercial radio-immunoassay (RIA) kit from Diagnostic Products Corporation (Los Angeles, USA). Interassay variability was 7.8% at a level of 25 IU/L. FSH was measured using the Enzymun-FSH test from Boehringer Mannheim GmbH, Diagnostica, Mannheim, Germany. Interassay variability was 3.7% at a level of 8.1 IU/L. Prolactin was measured using the Enzymun-Prolactin test (Boehringer Mannheim). Interassay variability was 5.1% at a level of 0,51 IU/L. Progesterone was measured using the Enzymun-Progesterone test (Boehringer Mannheim). Interassay variability was 2.5% at a level of 26 nmol/l. Estradiol was measured using the Coat-a-Count kit of Diagnostic Products Corporation. Interassay variability was 8.5% at a level of 976 pmol/l.

For statistical analysis we used the Mann-Whitney U test. Data are given as mean ± SEM. A P-value of $< 0.05$ was considered significant.

To calculate the proportion of all patients who had two cycles in which the progesterone level remained relatively low, the frequency was counted in two sample periods of six weeks (a 12-week period in total).

## Results

In the sample period of 12 weeks, 15 of 171 patients were identified whose progesterone values remained below the threshold of 32 nmol/l in two cycles, yielding a proportion of 8.8%. Fifty patients who met these criteria were included in the study. The patient characteristics are summarized in Table 2. In all patients except one, prolactin levels were found to be in the normal range (median 0.28, range 0.12 - 0.66; reference value <0.60 U/l). In all cycles, early

follicular FSH level was within the reference values of our laboratory (3-15 IU/l) with a median value of 5 IU/l and a range of 3-14 IU/l.

**Table 2** Patient characteristics of 36 with primary infertility and 14 with secondary infertility

| | *Range* | *Median* | *Mean* |
|---|---|---|---|
| Age (years) | 20-41 | 30 | 30.4 |
| Cycle length (days) | 24-33 | 28 | 27.7 |
| Infertility duration (months) | 12-137 | 30 | 48 |

Results of the 50 ECAs performed (Table 3) showed nine cycles to be completely normal according to the criteria applied. One of these cycles appeared to be a conception cycle. In 16 cycles ovulation occurred, but progesterone levels remained below the threshold of 32 nmol/l. In two cycles no follicle was seen; progesterone remained relatively low (16 and 18 nmol/l, respectively) in both cycles. Eight cycles showed an LH surge when a follicle that was too small (<17 mm at LH peak level) was observed. In all except one of these eight cycles, the progesterone level remained below 32 nmol/l. One of these patients conceived. She had a maximal mean follicular diameter of 13 mm and a peak progesterone level of 29 nmol/l before the hCG of the pregnancy could influence this value. Fourteen LUF cycles were seen. (luteinized

**Table 3** Typical features of the 'extended cycle analysis' for 50 cycles

| | | *Progesterone levels* (nmol/l) | | |
|---|---|---|---|---|
| Type of cycle | Number | Range | Median | Mean |
| Normal | 9 | 32-54 | 34 | 38.5 |
| Ovulatory, low progesterone | 16 | 10-31 | 27 | 26.4 |
| LUF cycle | 14 | 13-38 | 20 | 21.1 |
| Follicle < 17 mm at LH surge | 8 | 9-32 | 23 | 23.6 |
| No follicle at LH surge | 2 | 16-18 | 17 | 17.0 |
| No follicular growth | 1 | 1 | | |

LUF, luteinized unruptured follicle; LH, luteinizing hormone

**Table 4.** Clinical and hormonal characteristics in ovulatory cycles (groups 1 and 2 combined), cycles with luteinized unruptured follicles (group 3) and cycles with early onset of luteinizing hormone surge (group 4). Data are given as mean ± SEM

| | *Groups 1 and 2* | *Group 3* | *Group 4* |
|---|---|---|---|
| Age (years) | 31 ± 1 | 31 ± 1 | 27 ± 1* |
| Mean cycle length (days) | 28 ± 0 | 27 ± 1 | 28 ± 1 |
| FSH (IU/l) | 7.6 ± 0.6 | 7.0 ± 0.8 | 9.5 ± 1.6 |
| LH maximum (IU/l) | 60 ± 6 | 49 ± 6 | 48 ± 10 |
| Prolactin (U/l) | 0.33 ± 0.02 | 0.25 ± 0.03* | 0.28 ± 0.05 |
| Estradiol maximum (pmol/l) | 856 ± 40 | 866 ± 76 | 657 ± 111* |
| Progesterone maximum (nmol/l) | 31 ± 2 | 21 ± 2** | 20 ± 3** |

FSH, follicle stimulating hormone; LH, luteinizing hormone; *, $p < 0.05$; **, $p < 0.01$

unruptured follicle cycles were defined as normal follicular growth and normal hormonal events up to the moment of expected ovulation, but ovulation being absent). The remaining cycle showed no follicle growth or luteinization at all (progesterone < 3 nmol/l).

As the subjects in group 1 (normal cycle) and group 2 (ovulatory cycle but progesterone below 32 nmol/l) all showed normal follicular development and ovulation, we combined the data of these groups and compared them with the data of groups 3 (LUF cycles) and 4 (LH surge at a mean follicular diameter of < 17 mm) (Table 4).

In the 14 patients showing a LUF cycle, the peak plasma progesterone levels and prolactin levels were significantly lower. In the eight patients with an LH peak level at a follicular diameter of < 17 mm, significantly lower values of maximal estradiol, and progesterone were found, as well as a lower age. The significantly lower progesterone values in LUF cycles have been reported by others[6,7].

In summary, 50% of the observed cycles that were extensively analyzed showed definite abnormalities. As in 8.8% of all referred patients two low-progesterone cycles could be expected, an overall abnormality rate of at least 4.4% might be found in an infertile population.

## Discussion

In the present study we demonstrated that in 50 women with maximal luteal progesterone levels repeatedly below 32 nmol/l, only nine women (18%) had normal cycles according to prior criteria presented in Table 1. In 39 patients (78%) progesterone levels did not increase above 32 nmol/l. It is doubtful, if, notwithstanding a normal follicular growth pattern, normal ovulation and normal hormonal events, cycles may be considered as normal when mid-luteal progesterone levels do not reach the value of 32 nmol/l. If these cycles are considered as normal, 25 of the observed 50 cycles showed definite abnormalities. In 1978, Marik and Hulka[14] as well as the group of Koninckx[15] published their classical work on so-called LUF cycles: anovulatory cycles in regularly menstruating women, whose ovaries show corpus luteum formation and who have production of progesterone in the luteal phase. Koninckx and colleagues[15] suggested that LUF cycles could not be distinguished from ovulatory cycles by

hormonal parameters. Hamilton and co-workers[6], however, described a typical pattern of follicular growth (continuing growth instead of disappearance) in combination with a significant reduction of serum progesterone serum levels in the luteal phase, as well as LH peak values lower than those seen in control cycles. Striking were the normal follicular growth as well as hormonal events until LH reached its peak value. Ovulation, however, did not occur, and progesterone levels almost invariably remained below 32 nmol/l. Eissa and co-workers[7] gave the same definition for what they called 'cyst-like cycles' and they reserved the term 'LUF cycle' for cycles showing no rupture but some decrease in follicular volume after the LH surge, but with normal luteal phase progesterone. Hamilton and co-workers[9] observed 10 of 14 (71%) LUF cycles where progesterone did not reach 32 nmol/l, in contrast to no more than 10 of 127 (7.9%) abnormal cycles when progesterone was 32 nmol/l or more.

Several other more-or-less overlapping patterns of abnormal follicular growth have been described, indicating that there seems to be no typical feature, LUF being only one possibility[10,16-20]. Moreover, by studying subsequent cycles in the same individual, different patterns may be seen[7] , suggesting that the continuation of abnormal follicular growth is more important than the typical abnormalities observed.

It is not yet clear whether these cycles are associated with reduced fertility, or whether there is a causal relationship. Eissa and associates[7], however, found 58% abnormal cycles in an infertile group, compared with 23% in a donor insemination group ($p < 0.005$). Their group[7] and that of Hamilton[9] did not see any pregnancy in LUF cycles.

To be of significance for fertility, these abnormal cycles must occur in at least a substantial part of the cycles. The importance of these subtle ovulation disorders for fertility therefore remains questionable. Etiologic factors being unknown, there is no firm basis for any kind of treatment. Our preliminary results indicate that cycles are not always normalized, in spite of 'normalized' blood progesterone levels, with current ovulation-inducing treatments. We found persisting LUF cycles in two patients treated with anti-estrogens in spite of mid-luteal progesterone levels of 37 and 39 nmol/l. Another patient showed the same phenomenon during treatment with gonadotropins (human menopausal gonadotropin and hCG). Progesterone levels reached a value of 32 nmol/l.

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# Chapter 5

## DIAGNOSIS OF SUBTLE OVULATION DISORDERS IN SUBFERTILE WOMEN WITH REGULAR MENSTRUAL CYCLES: COST-EFFECTIVE CLINICAL PRACTICE?

P. van Zonneveld, H.P.F. Koppeschaar[1], J.D.F. Habbema[2], B.C.J.M. Fauser[3] and E.R. te Velde

Department of Reproductive Endocrinology and Fertility, [1]Department of Endocrinology, University Hospital Utrecht, [2]Department of Public Health, Erasmus University Rotterdam, and [3]Department of Obstetrics and Gynaecology, Dijkzigt Academic Hospital & Erasmus University Medical School, Rotterdam, The Netherlands

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## Abstract

Serial monitoring by plasma progesterone measurement is advised in the literature for fertility work-up, to detect ovulation disturbances in women presenting with regular menstrual cycles. Three strategies to diagnose such 'subtle ovulation disorders' (SOD, defined as anovulation, inadequately timed ovulation or ovulation of a follicle of reduced size in regularly cycling women) were evaluated, in order to investigate costs of such a diagnosis. On the basis of a 'maximal', an 'ultrasound-only', and a 'preselection' strategy, total medical costs and costs including non-medical costs were calculated for each SOD diagnosis. A 'maximal' diagnostic strategy resulted in a total medical cost of ECU 9057 per diagnosis (including non-medical costs ECU 12 787); an 'ultrasound-only' strategy in ECU 4520 ( ECU 6791) per diagnosis. By use of a 'preselection' strategy, 4.25% of the women were found to have an SOD, at a cost of ECU 3036 (ECU 6868) for each diagnosis. As the real significance of SOD diagnosis for the prognosis of the patient to become pregnant without treatment remains unclear, and as no randomized trials on treatment effectiveness have as yet been undertaken, it is questionable whether this approach is worthwhile.


## Introduction

In approximately 20% of couples who attend a fertility clinic, ovulation disorders associated with oligo- or amenorrhea are considered the most likely explanation for sub- or infertility[1]. Regularity of the cycle, and symptoms such as premenstrual breast swelling and dysmenorrhea, may be indicative of an ovulatory cycle. Recording of the basal body temperature (BBT) and progesterone measurements can be used for the qualitative or quantitative assessment of progesterone production. The use of serial hormone estimates in combination with ultrasound measurements has, however, shown that subtle ovulation disorders (SOD) may exist in a proportion of women presenting with regular menstrual cycles and a biphasic BBT[2,3]. Ovulation may either not occur at all, which is the case in luteinized unruptured follicle (LUF) cycles[2-5]. In these cycles a normal increase of serum estradiol concentrations is observed as well as a normal increase in follicle diameter. After the luteinizing

hormone (LH) surge, however, the follicle does not ovulate. Other instances of a SOD are an inadequately timed ovulation[6] (ovulation more than 48 h after the LH peak value) or ovulation of a follicle of reduced size (<17 mm in diameter)[6-8]. In cycles in which luteal serum progesterone concentrations remain relatively low, a high percentage of ovulation disorders has been described, especially LUF cycles[3,5]. Progesterone levels < 32 nmol/l may also have a predictive value for abnormalities in a subsequent cycle[9]. The use of serial hormone determinations together with repeated ultrasonographic measurements of follicle diameter is, however, a burden for the patient and is expensive. To our knowledge, a cost-analysis of such a diagnostic work-up has not been published to date.

The aim of the present study was to evaluate the costs necessary to diagnose subtle ovulation disorders, defined as anovulation or inadequately timed ovulation (ovulation of a follicle with a mean diameter < 17 mm or ovulation later than 48 h after the LH surge) in women with a history of regular menstrual cycles of 24 - 36 days' duration. We compared costs of diagnosing a subtle ovulation disorder between three diagnostic strategies: a most likely low-cost 'preselection' strategy, a 'maximal' strategy and an 'ultrasound-only' strategy.

## Materials and methods

### Description of the three diagnostic strategies

*Maximal diagnostic strategy*

Ideally, a diagnostic method identifies all fertility reducing factors in a particular patient group. With regard to subtle ovulation disorders this requires extensive cycle monitoring, combining hormone assays with sonographic findings in a so-called 'extensive cycle analysis' (ECA). If applied to all patients with a regular menstrual cycle of 24 - 36 days' duration, we called it the 'maximal diagnostic' strategy. Such a cycle analysis was carried out as follows. After determination of serum follicle stimulating hormone (FSH) on cycle day (CD) 1, 2 or 3, to exclude occult ovarian failure, serial ultrasound and hormone assays (luteinizing hormone (LH), 17ß-estradiol and progesterone) were performed every other day from CD 8 onwards, until a mean follicular

diameter of 13 mm was reached. Hereafter, daily examinations were performed until ovulation was observed, or until 3 days after the LH peak value if ovulation did not occur. In the remaining part of the cycle 17ß-estradiol and progesterone were measured at 2-3 day intervals until menses. Hormone assays were performed with commercially available kits, as described earlier[9]. We used preset criteria, based on the literature, to consider a cycle as normal: 17ß-estradiol rise in the late follicular phase > 400 pmol/l[10]; mean follicular diameter > 16 mm at the LH surge[6-8,11]; ovulation, defined as at least 60% decrease of mean follicular diameter, within 48 h after the LH surge[2,3,6,12,13].

*Ultrasound-only strategy*

With an 'ultrasound-only' approach, abnormalities such as an LUF cycle or ovulation of a follicle of reduced size would be seen, but hormonal events and the relation of these to follicular dynamics would remain obscure. As the occurrence of the LH surge as a reference point is not available, ultrasound scans will have to be continued for several days in cycles where ovulation does not occur at the expected time.

*Preselection strategy*

We applied a third option: a preselection of patients by screening for progesterone serum levels in the luteal phase. This option was based on a publication by Hamilton *et al.*[5] who found half of the cycle anomalies in a small group of women with progesterone levels < 32 nmol/l, whereas the other half was detected in the large group with normal progesterone. By serially measuring luteal progesterone levels, we tried to identify a group of patients who might have an increased chance to exhibit a subtle ovulation disorder in a subsequent cycle[9]. This part of the study was conducted at the Department of Reproductive Endocrinology and Fertility of the University Hospital Utrecht. In our infertility population, all women who had a regular cycle (24-36 days) were monitored with a basal body temperature (BBT) chart, while serum progesterone was measured at 3 -4 day intervals during the second half of the cycle. Progesterone was considered as normal if a level of 32 nmol/l was obtained on at least one occasion,[5,9,14]. If this value was not reached in 2

cycles, an 'extensive cycle analysis' (ECA) was performed. Fifty women who met these criteria were subjected to an ECA.
To calculate the proportion of all patients who had two cycles in which the progesterone level remained < 32 nmol/l, the frequency was counted in two sample periods of six weeks (a 12-week period in total). In this period of time, 177 couples of which the woman had regular cycles were referred to our fertility unit.

## Calculation of the costs

Costs can be divided into direct medical costs (wages for medical and laboratory personnel, expenses for analyser machines and reagents, additional costs for housing, energy and maintenance), indirect medical costs (personnel for administration and desk tasks, expenses for computers, telephone costs, housing and maintenance), and non-medical costs (costs patients have to make to visit the hospital, loss of productivity when traveling to and staying at the hospital). In our calculations we restricted ourselves to the costs during the diagnostic process. Direct and indirect medical costs (total medical costs) are given, and where appropriate an indication of total cost (total medical costs and non-medical costs) is mentioned. The values of these costs are derived from an investigation of real costs of diagnostic procedures in the University Hospital Utrecht which was performed in 1991 by the Department of Administration and Information, and not from the negotiated tariffs, charged to the patients, for laboratory and ultrasound procedures[15]. In this investigation the non-medical costs were also calculated for an average Dutch population. We must realize, however, that non-medical costs may differ greatly between countries and patient populations. In our patients, the mean time period needed for traveling, waiting and blood withdrawal was estimated to be 84 min; for an ultrasound examination this time period was 79 minutes. Costs for loss of production were considered ECU 11.7/h; for each hospital visit an amount of ECU 9.15* was calculated as costs for transportation.

*1 ECU = £0.65.

## Results

### Costs as calculated for the three strategies.

The medical costs for the applied hormone assays and transvaginal ultrasonography are given in Table 1. The total medical costs for the three strategies were calculated as described below.

**Table 1** Medical costs for some hormone assays and transvaginal ultrasonography.
The costs are given in ECU (1 ECU = Dfl 2.23 = £ 0.65)

| *Investigation* | *Direct medical costs* | *Indirect medical costs* | *Total medical costs* |
|---|---|---|---|
| LH | 5.54 | 5.34 | 10.88 |
| FSH | 4.64 | 5.34 | 9.98 |
| 17b-Estradiol | 13.01 | 4.74 | 17.75 |
| Progesterone | 5.87 | 5.34 | 11.21 |
| Ultrasound | 13.83 | 34.20 | 48.03 |

*Maximal diagnostic strategy*

A cycle of every patient should be examined in a precise way, as we did in our selected patient group (see *Preselection strategy*). In our investigation the mean number of hospital visits for the screening of one cycle was 13, during which seven ultrasound examinations, one FSH, seven LH, 12 17ß-estradiol and 12 progesterone measurements were performed. One hundred extended cycle analyses (ECA) would cost (total medical costs) ECU 76 987. In our patient population the extra non-medical costs were ECU 31 700. Costs for each SOD diagnosis would depend on the incidence. With the preselection approach we found an incidence of 4.25%. On the basis of the study of Hamilton *et al.*[5] we assumed that the incidence in the whole population is twice as high (8.5%). Thus, it can be calculated that the total medical costs of diagnosing one SOD would be ECU 9057 (total costs ECU 12 787).

*Ultrasound-only strategy*

Using the 'ultrasound-only' strategy in all patients, and assuming eight ultrasounds per cycle, for 100 cycles the total medical costs would be ECU 38 424 (non-medical costs a surplus of ECU 19 300). If all disorders were to be detected, again assuming an incidence of 8.5%, the total medical costs for one diagnosis would be ECU 4520; the costs including non-medical costs ECU 6791.

*Preselection strategy*

In the sample period of 12 weeks, 177 women with regular cycles were eligible for screening for luteal progesterone. Progesterone serum values were = 32 nmol/l in 145 women (82%) and < 32 nmol/l in 32 (18%). Progesterone measurements were not repeated In eight patients because of severe sperm abnormalities, tubal disease or because the patient was unable to attend the outpatient clinic regularly. In 24 patients, progesterone measurements were repeated in a subsequent cycle. In nine of these, a progesterone value of ≥ 32 nmol/l was found (5% of the initial patients), while in 15 patients (8.5%) the progesterone level was again < 32 nmol/l. In an earlier study[9], we performed

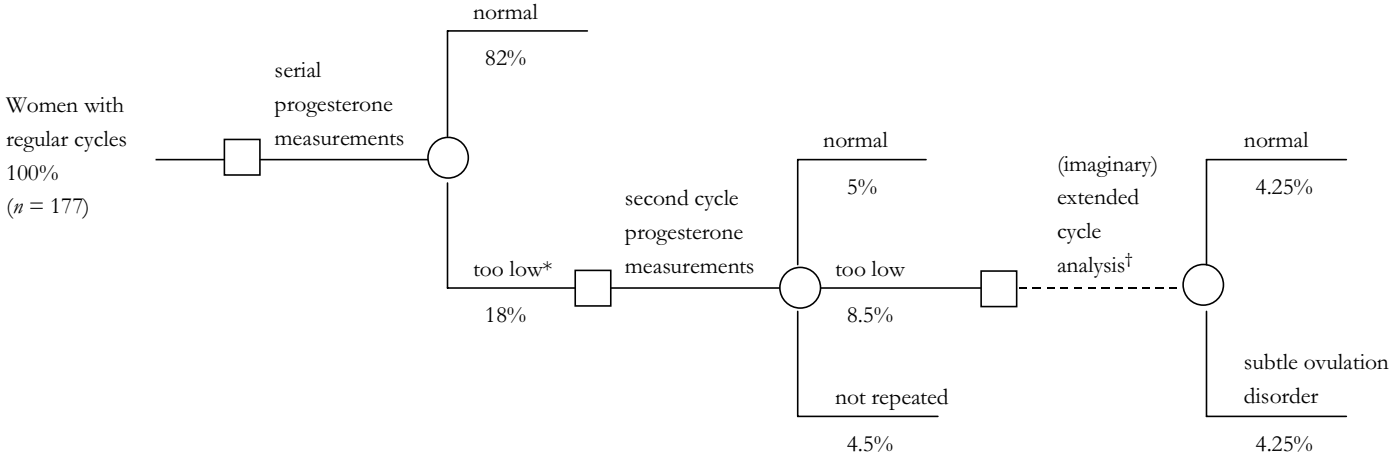


**Figure 1.** Combined results of serial luteal phase progesterone measurements in 177 patients, and extensive analysis of a cycle in 50 patients. *Defined as all progesterone concentrations < 32 nmol/l when measured at 3-4-day intervals in the second half of a cycle. †In 50 cycles, preselected in the same way, a 50% incidence of subtle ovulation disorders was found

an extensive cycle analysis in 50 patients who had had a low-progesterone cycle twice before. We found a subtle ovulation disorder in 50%. The combined results of the above mentioned observations are summarized in Figure 1. Numbers are given as the percentages of the initial number of individuals ($n$ = 177).

For calculation of the costs, we started with an imaginary cohort of 100 patients. The mean number of visits needed to perform the serial progesterone measurements was five. In the extensive cycle analysis, the mean numbers of the various assays and investigations have been mentioned above. The financial costs were calculated on the basis of the foregoing assumptions. Of the initial 100 serial progesterone determinations, 13.5 had to be repeated. The total medical costs for these prescreening cycles would be ECU 6362. Extensive cycle analysis would have been done 8.5 times at a total medical cost of ECU 6544. For the 100 referred patients costs would be ECU 12 906 (non-medical costs ECU 18 000 extra). In this group of patients a number of 4.25 subtle ovulation disorders are expected. This implies that the total medical costs of diagnosing one case would be about ECU 3036 (total costs ECU 6868).

## Discussion

Monitoring of plasma progesterone is generally advised for fertility work-up to detect ovulation disturbances in women presenting with regular menstrual cycles. Reported minimal progesterone levels to confirm ovulation range from 9.6 to 32 nmol/l. Andrews[16] mentions a lower limit of 9.6 nmol/l, Glass[17] 9.6-12.8 nmol/l, Greenspan[18] 12.8 nmol/l, Rowe *et al.*[19] 18 nmol/l, and Speroff[20] 21 nmol/l, preferably 32 nmol/l. In the recently published guidelines of the ESHRE Capri Workshops[21], progesterone concentrations > 16 nmol/l for a minimum of 5 days are suggested as indicative for ovulation. This recommendation is based on one report by Landgren *et al.*[22]. In this investigation of 68 normally menstruating women, 94% appeared to have blood serum progesterone concentrations > 16 nmol/l for at least 5 days. The above-mentioned cut-off values were chosen as the authors arbitrarily expected 95% of all women to have ovulatory cycles. The ESHRE Group[21] advocates the measurement of progesterone as 'the most cost-effective compromise' in diagnosing anovulation, an

ideal method being absent. In the above-mentioned publications, no references to any published results of research are given, except for the study of Landgren *et al.*[22], in which their unproven hypothesis was used to determine a progesterone level that distinguishes an ovulatory from an anovulatory cycle. None of the publications provides guidelines as to how to confirm a diagnosis of anovulation when too low progesterone concentrations have been found. Also, data on recurrence of low-progesterone cycles and on the prognosis for pregnancy without treatment[23] once a low-progesterone cycle has been established, are not given. Recommendations for treatment, if mentioned, include the use of various drugs for ovulation induction. Although pregnancies have been documented following such treatments[4,24-28], none of these studies are based on randomized controlled trials.

In our patient population, screening by serial progesterone measurements, and subsequently performing an extended cycle analysis in cases where no progesterone value was > 32 nmol/l in two cycles, resulted in a 4.25% incidence of the diagnosis 'subtle ovulation disorder'. This 'preselection' strategy appears to entail less than half the total medical costs of the 'maximal' strategy and less than the costs of the 'ultrasound-only' strategy. By using the 'preselection' strategy, however, about half of the diagnoses SOD will remain unidentified, and with the 'ultrasound-only' strategy some cases may be missed. With the latter strategy, no LUF cycle, an otherwise anovulatory cycle, or a cycle in which a too small follicle ovulates, would escape attention, but other subtle ovulation disorders, for instance delayed ovulation, cannot be diagnosed in this way. In the 50 cycles we observed[9], 25 were abnormal. These cycles would have been adequately diagnosed with ultrasound alone.

Although the 'preselection' and ultrasound strategies are the least expensive approaches, they still result in high costs for establishment of diagnosis. In our opinion, these costs are not in balance with the possible but still unclear and unproven advantages. Regardless of how subtle ovulation disorders are diagnosed, treatment strategies with proven efficacy are lacking.

Nevertheless, measuring of progesterone concentrations in the diagnostic work-up of women who attend a fertility clinic is common practice. This indicates that we are still largely in the era of 'authority-based' rather than 'evidence-based' medicine. We make great efforts to diagnose a disorder of which we do not know whether or not it is a repetitive phenomenon, and whether or not its

treatment is effective.

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# Chapter 6

## HORMONE PATTERNS AFTER INDUCTION OF OVULATION WITH CLOMIPHENE CITRATE: AN AGE-RELATED PHENOMENON

P. Van Zonneveld, G. Scheffer, H.P.F. Koppeschaar*, B.C.J.M. Fauser**, F.J. Broekmans and E.R. te Velde

Department of Fertility and Reproductive Medicine, Division of Obstetrics and Gynaecology, *Department of Endocrinology, University Hospital Utrecht and **Department of Obstetrics and Gynaecology, Dijkzigt Academic Hospital and Erasmus University Medical School, Rotterdam, The Netherlands

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## Abstract

Since the introduction of Clomiphene citrate (CC), more than three decades ago, a discrepancy has been observed between ovulation and pregnancy rates for which as yet no explanation exists. To investigate if ovulation disorders or abnormal hormonal patterns occur more often in CC-stimulated seemingly ovulatory cycles, we performed hormonal and sonographic monitoring in first cycles of oligo- or amenorrheic patients who were stimulated with 50 mg CC, and compared the hormonal patterns to these in natural cycles of age-matched proven fertile women. Twenty-four first CC cycles were monitored. Twelve cycles appeared to be ovulatory, eleven showed no follicle development and one cycle exhibited the luteinized unruptured follicle (LUF) phenomenon. Ten ovulatory cycles were compared with 27 unstimulated control cycles. In four cycles stimulated by CC, a temporary decline in estradiol levels was apparent. In these cycles estradiol reached a higher level on cycle day (CD) 7 or 8 compared to cycles without a decline. Such an estradiol decline was seen in only one control cycle. Furthermore, the estradiol levels on CD 7 or 8 appeared to be age-related.

We conclude that the estradiol decline in CC-stimulated ovulatory cycles may be a consequence of a sharp rise after CC stimulation, and such a rise may be age-related and coincide with a diminished follicle quality. If this phenomenon is associated with a suboptimal cycle, and so contributes to the suboptimal pregnancy rates after ovulation induction treatment with clomiphene citrate, is still unknown.


## Introduction

Clomiphene citrate (CC) has been used since 1967 to induce ovulation in patients with oligo- or amenorrhea who wish to become pregnant. It is generally accepted as the first-line drug in normogonadotropic patients including patients with the polycystic ovary syndrome (PCOS). The mechanisms of action of CC are not fully understood[1,2]. CC is able to occupy estrogen receptors for a prolonged period of time and it has agonistic as well as antagonistic properties. CC is thought to act mainly by (partially) blocking the negative feedback of 17ß-estradiol in the hypothalamic-pituitary axis. CC administra-

tion is followed by a rise in serum concentrations of both luteinizing hormone (LH) and follicle-stimulating hormone (FSH)[3,4], subsequently stimulating follicular growth.

A concern regarding CC treatment is the discrepancy between ovulation rates and pregnancy rates. Of normogonadotropic and PCOS patients 57-91% will ovulate as judged by a biphasic basal body temperature (BBT) record, a progesterone rise or a secretory endometrium, but only 25-43% become pregnant[5-7]. Several factors have been implicated for this discrepancy including other causes for the infertility, endometrium receptivity, corpus luteum function, early embryo quality and cervical mucus[6]. Little is known about disorders of follicle growth and ovulation in patients treated by CC for anovulation or oligo-ovulation. Hamilton *et al.*[8] observed six luteinized unruptured follicle (LUF) cycles in 45 CC-stimulated cycles. In studies comparing hormonal patterns in CC cycles and spontaneous cycles, no reasons for concern have been found[3,4,9]. However, except for the very first studies, results are given as mean values of groups of patients and not of individual patients. In two studies[3,10] where individual hormone patterns are shown, a temporary decline of serum estradiol levels can be seen. No attention to this phenomenon has been paid. To investigate if ovulation disorders like LUF cycles, or abnormal hormonal patterns occur more often in CC-stimulated seemingly ovulatory cycles, we performed hormonal and sonographic monitoring in first cycles of oligo- or amenorrheic patients who were stimulated with 50 mg CC. We compared the hormonal patterns with these in natural cycles of age-matched proven fertile women.

## Subjects and study design

The study was conducted at the Department of Reproductive Endocrinology and Fertility of the University Hospital Utrecht. From august 1995 we offered all patients who were treated with CC, hormonal and ultrasonographic monitoring of the first cycle. The reason for this was the uncertainty regarding the discrepancy of ovulation and pregnancy rates as mentioned before. Ovulation disorders such as LUF cycles, or abnormal hormonal patterns could be responsible for this problem. We felt that monitoring of the first cycle might guard patients for several months of inadequate treatment.

Twenty-four patients (age 22-33 years), treated from August 1995 until July 1996 were included in the present study. Five of them were amenorrheic, 18 patients had oligomenorrhea (mean cycle length > 6 weeks) and in one patient with otherwise unexplained infertility a LUF cycle had been observed. Fourteen patients were diagnosed as PCOS on the basis of an elevated (> 10 IU/l) serum LH and/or elevated testosterone (>2.0 nmol/l) or androstenedione (>7.0 μmol/l) concentrations. Six patients had a normogonadotropic and normoestrogenic hormonal status and three patients had a normogonadotropic and hypo-estrogenic amenorrhea. The latter group of patients is always offered a 'try-out' treatment with CC in our department.
The control group consisted of 27 healthy volunteers in the same age group (22-33 years), who took part in a large study on the effects of age on ovarian function. Volunteers were enrolled in the study protocol if they met all of the following criteria: (a) regular menstrual cycles with a length between 23 and 35 days, (b) biphasic BBT, (c) proven natural fertility by having had at least one pregnancy, (d) each of the pregnancies had to be established within one year of exposition, (e) no evidence of any endocrinological disease, (f) no history of ovarian surgery, (g) no ovarian abnormalities as assessed by vaginal ultrasound, (h) interruption of hormonal contraception at least 2 months before entering the study protocol, (i) written informed consent given.

## Ovulation induction and monitoring

The patients were treated with 50 mg CC on the third to seventh day after the start of a spontaneous or a progestogen-induced vaginal bleeding. Monitoring consisted of repeated serum estradiol, FSH, LH and progesterone measurements, and ovarian ultrasonography. Only the first induction cycle of each patient was used for evaluation. One cycle of each control woman was used for comparison. The same parameters were used.
Hormone assays were performed with commercially available kits. Estradiol concentrations were assayed with a MEIA from Abbott Laboratories (Abbott Park, IL, USA) which was performed on the semiautomated IMx analyzer. Between-run coefficients of variation were respectively 10.1; 7.0 and 6.9% at 533; 1354 and 4197 pmol/l (n=49, 49 and 30). Serum concentrations of FSH were measured by the Enzymun-FSH test (Boehringer Mannheim, Mannheim,

Germany) on the automated immunoanalyser ES-600. This assay is calibrated against the WHO Second International Reference Preparation for human FSH (83/575). The observed interassay coefficients of variation (CV) were 3.7; 2.3 and 2.7% respectively at 8.1; 22 and 75 IU/L (n=27). For LH a similar test was used, calibrated against the second IRP 80/552. For LH interassay coefficients of variation of 2.4% at 6.1 U/l; 5.1% at 11.5 U/l and 6.7% at 23.2 U/l were found (n=17). For ultrasound a Toshiba SSH-140 A or a Toshiba Capacee (Nasu-Works, Nasu, Japan) real-time sector scanner was used, employing a 5-mHz transvaginal transducer, calibrated at a sound velocity of 1510 m/s. Of the follicles = 8 mm, the largest diameter and the diameter perpendicular to this one were measured. From these two diameters, the mean diameter was calculated and considered as the mean follicular diameter.
Monitoring was carried out on cycle day (CD) 3, 7 and thereafter every other day until the largest follicle measured 14 mm. Subsequently, the patients were seen daily until ovulation had occurred (total collapse of the follicle or partial collapse by 50% or more compared to the previous mean diameter[11]) or until 3 days after the peak LH value. The control women were investigated on CD 1, 2 or 3, thereafter on CD 5, 6 or 7 and subsequently, every other day and in the same way as the patients.

## Methods of analysis

Results of hormone assays are influenced by the interassay variation of the used method and by within-subject biological variation of hormone levels. Because we wanted to analyze hormone patterns of individual patients, the so-called 'critical difference' between hormone levels at different times was calculated. For this purpose we applied the following formula[12]:

$$CD = Z \times \sqrt{2} \times \sqrt{(CV_A^2 + CV_I^2)}$$

where CD is expressed as a percentage; $CV_A$ is the analytical imprecision, $CV_I$ is the average inherent within-subject biological variation, and Z is the Z-score which depends on the probability selected for the statistical significance. At the 0.95 confidence level, Z = 1.96. In our calculation we used 1.96 for Z; 10% for $CV_A$ (interassay coefficient of variance of the assay), and considering the patients their own control 0% for $CV_I$. A difference of > 27.4% resulted as a difference at the 95% level, so estradiol concentrations were considered to be

declined compared to a previous one, if the difference between the two values exceeded 27.4%.
Data were analysed using SPSS (version 6.4, for Windows): Fisher's Exact Test for comparison of proportions, the Mann-Whitney U Test for comparison of groups of samples, and correlation tests. A probability of $< 0.05$ was regarded as significant.

## Results

Twenty-four patient cycles and 27 control cycles were monitored. Twelve of the CC-induced cycles appeared to be ovulatory, eleven showed no follicular development nor estradiol rise, and one LUF cycle was seen. PCOS patients more often had an anovulatory cycle (9 out of 13) than patients with other diagnoses (2 out of 10; P=0.04, Fisher's Exact Test). The mean age (± SD) of all CC-treated patients was 27.8 ± 3.6 years. The patients with an ovulatory cycle were 28.3 ± 3.6, and the patients with an anovulatory cycle were 27.7 ± 3.3 years old. The patient who had a LUF cycle was 22 years old. The mean age of the volunteer women was 29.1 ± 2.8 years (differences non-significant).
The patterns of follicle growth and the concentrations of estradiol, FSH and LH in the 12 ovulatory CC cycles were compared with those in ovulatory cycles of the 27 volunteer women. In line with data reported in the literature, mean estradiol concentrations were higher during the midfollicular phase in the CC-stimulated cycles, as were FSH and LH (results not shown). The mean follicle diameters were not different in CC compared with control cycles.
Mean or median values of pooled cycles, however, do not give information about hormone levels or follicle growth *in individual patients*. Therefore we studied the patterns of the estradiol concentrations in the follicular phase of individual cycles in detail, and we compared them with those in ovulatory cycles of the 27 volunteer women. In two CC cycles this was not possible because both on CD 7 and CD 8 no blood test results were available.
Of the ten evaluable ovulatory cycles after 50 mg of CC per day during CD 3-7, four showed a temporary decline of serum estradiol concentrations between CD 7 and 11 by more than 27.4% (the 'critical difference'); in three other cycles a decline also occurred, however, by 10.9%, 20.5% and 22.1% respectively. In Figure 1, the estradiol levels of each of the four cycles express

ing a decrease >27.4% are shown. The mean magnitude of the decline was 44.6% (range 28.8-64.5%). After this decline, the estradiol levels showed a continuous rise until the start of the pre-ovulatory LH surge. In three of these cycles the follicles showed a steady increase in size, in one the mean follicle diameter remained 14 mm during CD 6-10: thereafter, it increased to 26 mm before ovulation. The duration of the follicular phase, the growth rate of the follicles, and the maximally reached follicular diameters before ovulation were not different between the two groups.

Of 27 control cycles, a decline in estradiol concentration was only observed in one cycle. In this cycle, a follicle was observed on CD 9, that reached a diameter of 13 mm and disappeared after CD 12, on which day another follicle took over. The estradiol level fell by 36.4%.

Summarizing in 4 of 10 CC-stimulated cycles, an estradiol decline of > 27.4% occurred, as compared to 1 of 27 unstimulated control cycles ($p$ = 0.01, Fisher's Exact Test; odds ratio 17.3 with 95% confidence interval 1.6-184.4).

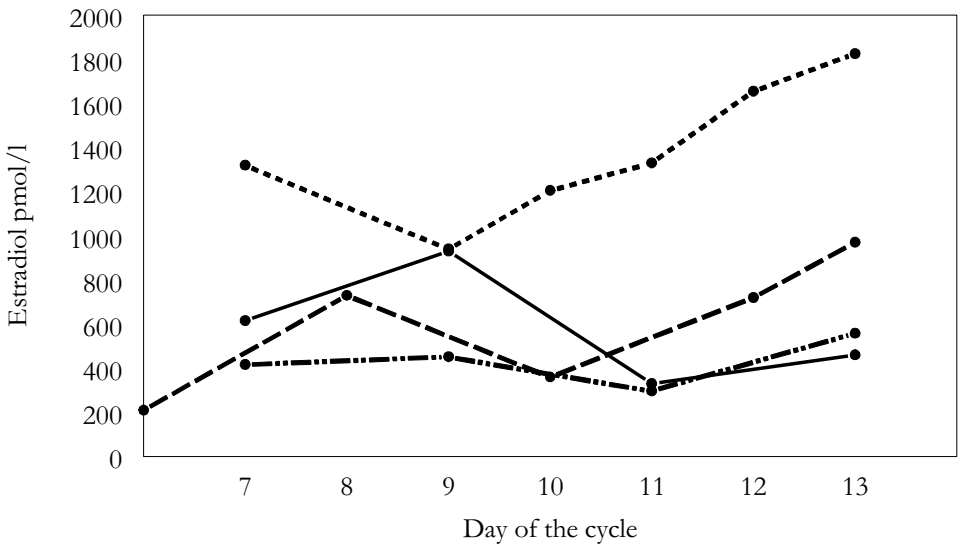


**Figure 1.** Serum estradiol concentrations in the follicular phase of four cycles expressing a temporary decline > 27.4%

To compare estradiol concentrations between the two groups of CC-stimulated cycles (with or without estradiol decline) and the control cycles, estradiol levels of 2 consecutive days were clustered because the patients as well as the control women were seen on alternate days. If estradiol was determined on both days, the mean of the two concentrations was used. estradiol concentrations on CD 7-8 are shown in Figure 2. As expected, estradiol concentrations were higher in the whole group of CC cycles compared to control cycles ($p < 0.01$; result not shown in the figure). In the four cycles in which estradiol declined beyond the 'critical difference', estradiol was higher than in cycles that showed no such decline ($p = 0.05$) (see Figure 2). Similar trends were seen for FSH concentrations ($p < 0.01$ for cycles with, and $p = 0.14$ for cycles without significant estradiol decline respectively).

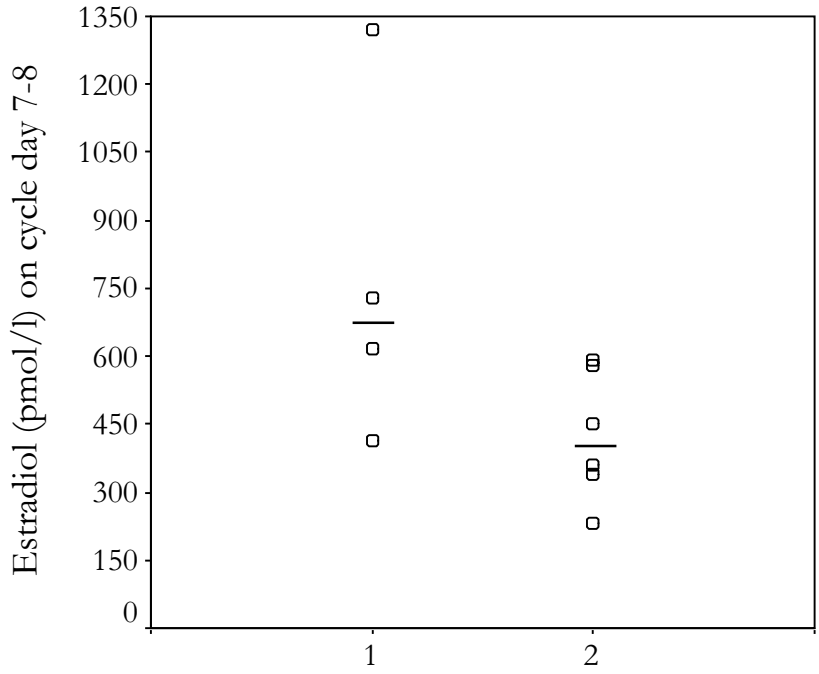


**Figure 2.** Serum estradiol concentrations on cycle day 7 or 8 in clomiphene citrate-stimulated patients. Group 1 with estradiol decline > 27.4%; group 2 without decline. Median values are indicated

To investigate if higher estradiol concentrations on CD 7-8 correlated with age, these parameters were plotted against each other (Figure 3). A positive correlation was observed between age and estradiol level in the 10 CC-treated cycles (Pearson's $r = 0.67$; $p < 0.05$). Similar correlations were found within both subgroups. The *maximum* estradiol concentration that was reached in each of the CC-stimulated cycles was also plotted against age; no correlation appeared to be present. FSH concentrations on CD 7-8 did not appear to be related to age. In the control group non-stimulated estradiol levels did not appear to be correlated with age (data not shown).

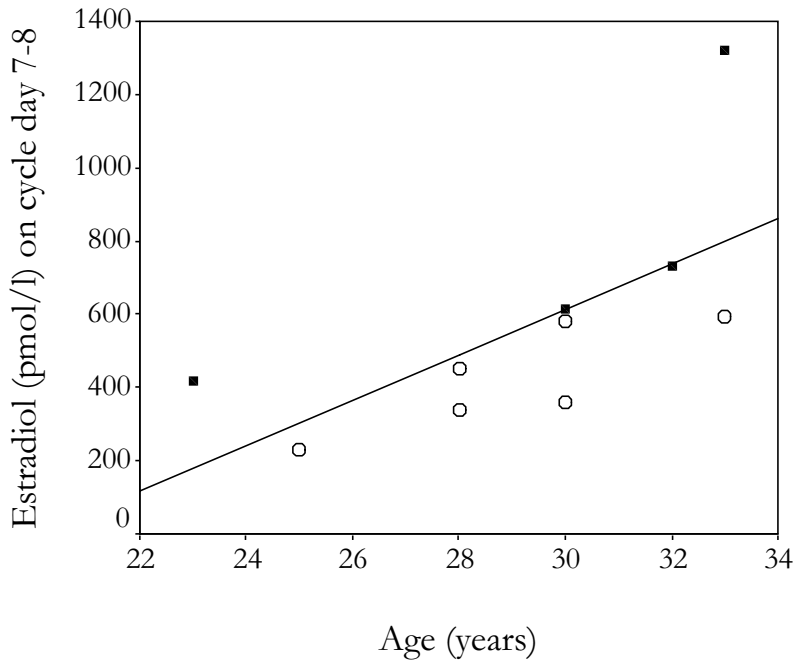


**Figure 3.** Relation of serum estradiol on cycle day 7 or 8 to age in ten clomiphene citrate-stimulated patients. Group 1 with estradiol decline > 27.4% (■), group 2 without estradiol decline (O).For the total population, $r = 0.67$ and $p < 0.05$

**Discussion**

In 24 first CC-stimulated cycles, 12 appeared to be ovulatory, 11 anovulatory, whereas 1 LUF cycle was seen. A 50% ovulation rate in a first CC cycle is in agreement with literature data[13]. Ovulation disorders such as LUF (1 out of 24 cycles) can not be considered as an explanation for the reported discrepancy between the ovulation rate and the pregnancy rate in CC cycles. In the follicular phase of ovulatory CC-stimulated cycles a temporary decline in estradiol levels apparently often occurs, in contrast to natural cycles (4 out of 10 cycles, compared to 1 out of 27 unstimulated control cycles). This phenomenon has previously been shown, but its significance has not been subject of discussion. This decline may be explained by a high response of estradiol production during the CC exposure. In the four cycles with an estradiol decline, the estradiol concentrations were indeed higher on CD 7-8 compared with cycles without a decline and control cycles. The same trend was observed for FSH. There appeared to be a positive correlation of estradiol levels and age.

These observations might be explained by the findings of Hughes *et al.*[14]. They examined serum inhibin and estradiol responses to ovarian hyperstimulation for *in vitro* fertilization. Inhibin responses were significantly lower in women 35 years of age or older. Estradiol responses were not influenced by age. This suggests that the two hormones reflect different granulosa cell functions, and that a diminished inhibin response to ovarian stimulation may be a marker of declining granulosa cell function with age. In other words: as the ovarian depletion of follicles progresses, the production of inhibin is impaired; however, the production of estradiol is not yet impaired at an early stage of follicle depletion.

If we apply the findings of Hughes *et al.* to our patients, we come to the following hypothesis: After the production and release of FSH is increased by the action of CC, the negative feed back by inhibin towards the central system is subnormal in the older patients. As a result the FSH concentrations rise to a higher level. This higher concentration of FSH stimulates the estradiol production of the cohort of follicles that had been recruited at that moment. The rise of estradiol prevents the FSH levels from rising above the normal range by activating estradiol dependent negative feed back mechanisms. The net result is a higher estradiol level at the end of the stimulation by CC, on CD 7-8.

Hereafter, the selection of a dominant follicle takes place and the estradiol levels at the end of the follicular phase no longer reflect an age-dependancy. The significance of these phenomena for the quality of the cycle, however, remains unknown, and there is as yet no proof to suggest that they indicate suboptimal follicle growth, and so contribute to the discrepancy between the ovulation rate and the pregnancy rate in patients using CC for ovulation induction. We conclude that the often occurring temporary decline of estradiol at the midfollicular phase is an age-dependent phenomenon.

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# Chapter 7

## DO CYCLE DISTURBANCES EXPLAIN THE AGE-RELATED DECLINE OF FEMALE FERTILITY? CYCLE CHARACTERISTICS OF WOMEN ABOVE 40 COMPARED TO A REFERENCE POPULATION OF YOUNG WOMEN

P. van Zonneveld[1], G.J. Scheffer[1], F.J.M. Broekmans[1], M.A. Blankenstein[2,5], F.H .de Jong[3], C.W.N. Looman[4], J.D.F. Habbema[4] and E.R. te Velde[1]

[1]Department of Reproductive Medicine, Division of Obstetrics, Neonatology and Gynecology, [2]Department of Endocrinology, University Medical Center Utrecht, Heidelberglaan 100, 3584 CX Utrecht, [3]Department of Internal Medicine and [4]Department of Public Health, Erasmus University Medical Center Rotterdam, [5]Present address: Department of Clinical Chemistry, VU University Medical Center, Amsterdam

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## Abstract

BACKGROUND: The cause of declining fertility with age, in women who still have regular menstrual cycles, is not clear. METHODS: We evaluated follicle development, endometrial growth and hormonal patterns in cycles of older women (age 41-46 years, n = 26) who were normally fertile in the past, and compared these cycles with a reference group of relatively young fertile women (age 22-34 years, n = 35). RESULTS: Clearly abnormal cycles were found in only two women in the older age group, compared to one in the younger group. The main differences between the age groups were a shorter follicular phase and cycle length in the older group, in combination with higher FSH levels in the late luteal and early follicular phase. In contrast to literature data which suggest an 'accelerated' follicle development in older women, we found sonographical and hormonal evidence of an 'advanced' follicle growth, with an earlier start already during the luteal phase of the preceding cycle, and an advanced selection and ovulation of the dominant follicle. CONCLUSIONS: Such an earlier start of follicle growth in a possibly less favourable hormonal environment, as well as a limited oocyte pool, may contribute to a decreased follicle and oocyte quality, resulting in diminished fertility in aging women.


## Introduction

On average, female fertility declines from age 30 onwards (van Noord-Zaadstra *et al.*,1991) or even earlier (Leridon, 1977). In so-called natural breeding populations when no contraceptive measures are used, the end of fertility is reached at a median age of 40-41 years (Bongaarts, 1982; Wood, 1989). Menstrual cycles, however, continue to be remarkably regular until a mean age of 45-46 (Treloar, 1981) and it takes another 5 years before menopause is reached (den Tonkelaar *et al.*, 1998). Postponement of childbearing, which is a demographic trend in all Western countries, considerably contributes to the increasing proportion of subfertile couples (Mosher and Bachrach, 1996). The cause of declining fertility in elderly women who still have regular menstrual cycles, is not clear. Possible explanations include a higher incidence of subtle cycle disorders such as anovulatory or luteinized unruptured follicle (LUF) cycles, hormonal changes, diminished uterine receptivity, or an oocyte factor. Age-

dependent hormonal changes have been described by several authors. The earliest endocrine change associated with reproductive aging is a selective rise of FSH in the early follicular phase from age 35-40 onwards (Sherman *et al.*, 1976; Reyes *et al.*, 1977; Musey *et al.*, 1987; Lee *et al.*, 1988; Fitzgerald *et al.*, 1994; Klein *et al.*, 1996a). A rise of LH also occurs, but 5-10 years later (Ahmed Ebbiari *et al.*,1994). The available data about estradiol and progesterone do not show a consistent pattern. It has been suggested that the rise of FSH is primarily caused by a decreased secretion of ovarian inhibin. Klein *et al.* (1996b) showed a negative association between FSH and inhibin B in the early follicular phase, but could not demonstrate such an association for inhibin A. A probable explanation is that inhibin A is synthesized by the dominant follicle and the corpus luteum, whereas inhibin B is secreted by granulosa cells of developing antral follicles. Evidence is accumulating that a selective rise of FSH is due to diminished negative feedback by inhibin B during the early follicular phase (Groome *et al.*, 1996; Klein *et al.*, 1996b) and by inhibin A during the preceding luteal phase of the cycle (Reame *et al.*, 1998; Danforth *et al.*, 1998; Welt *et al.*, 1999; Santoro *et al.*, 1999), acting in a tandem fashion to restrain FSH secretion (Santoro *et al.*,1999).

Although much is known about hormonal patterns and follicle development in the normal menstrual cycle, it is difficult to define cycle abnormalities unless there is no follicle growth at all or no ovulation. In the present study we used observations in young and fertile women as a reference of cycle normality. We considered the cycles of these women as normal, unless definite abnormalities would be seen such as absence of follicle development or ovulation.

The aims of the present study are:

1. To obtain reference values for cycle normality from a group of relatively young fertile women who have regular menstrual cycles.
2. To evaluate whether follicle growth, ovulation, hormonal patterns and endometrial development in older women differ from this reference group, and, if present, whether such differences may explain the age-related loss of fertility.

## Materials and methods

*Subjects*

The study was conducted at the department of Reproductive Endocrinology and Fertility of the University Medical Center Utrecht. It was approved by the local ethics committee, and written informed consent was obtained from all participants. Healthy women (aged 22-46 years) were recruited by advertisement in local newspapers. Volunteers were enrolled in the study if they met the following criteria: (1) regular menstrual cycles varying from 21 to 35 days, (2) biphasic basal body temperature (BBT), (3) proven natural fertility by having had at least one pregnancy, (4) each of the pregnancies had to be established within one year after discontinuing contraception, (5) no evidence of endocrinological disease, (6) no history of ovarian surgery, (7) no ovarian abnormalities as assessed by vaginal ultrasound, and (8) cessation of hormonal contraception at least 2 months before entering the study protocol. The volunteers received monetary compensation for study participation.

*Experimental design*

A relatively older group of women who were normally fertile in the past (n = 26, age 41-46 years), was compared to younger women, the reference group (n = 35, age 22-34 years). The investigations started in the midluteal phase of the first study cycle. The luteal phase was assumed to have started when a temperature rise on the BBT chart had been observed. The temperature was considered to be elevated if it was higher than the temperature on any of the six preceding days (World Health Organization, 1967). From the seventh day after the temperature shift, the volunteers visited our research department every two or three days for ultrasound (US) scans in which the size and number of all follicles 2 mm in diameter or above, were counted and measured, and endometrial thickness was determined. Blood samples were taken at each visit and stored for determination of follicle stimulating hormone (FSH), luteinizing hormone (LH) and estradiol ($E_2$). In the samples which were most close to the start of the menstrual period, inhibin A and B were also determined. After the menstruation had started in the subsequent cycle (cycle 2) the

volunteers returned on cycle day 2, 3 or 4, for US, $E_2$, FSH,LH, inhibin A and B. Thereafter, every two or three days the same measurements except inhibin A and B were performed. When the dominant follicle had reached a mean diameter of at least 14 mm, US scans were performed and blood samples were taken daily until ovulation had occurred. Ovulation was defined as a complete disappearance of the follicle or a reduction of its mean diameter by at least 5 mm (Janssen-Caspers *et al.*, 1986; Check *et al.*, 1990). After ovulation an US scan was performed in the midluteal phase. At the same time blood was taken for measuring progesterone. The length of the follicular phase was defined as the period of time (in days) from and including the day the menstrual period started, until and including the last day the dominant follicle was present before ovulation. The length of the luteal phase was considered the interval following ovulation up to and including the day before the onset of menstruation.

*Hormone assays*

Blood sampling and transvaginal sonography were performed on the same days. Hormone concentrations were measured in plasma ($E_2$, FSH, LH and progesterone) and serum (inhibin A and B) specimens stored at -20° C until processing. Estradiol concentrations were assayed with a Microparticle Enzyme Immunoassay (MEIA) from Abbott Laboratories (Abbott Park, IL, USA) which was performed on a semiautomated IMx analyzer. Between run coefficients of variation for $E_2$ were 10.1; 7.0 and 6.9% at 533; 1354 and 4197 pmol/l, respectively (n=49; 49 and 30). Concentrations of FSH, LH and progesterone were measured with the fully automated AxSYM immunoanalyzer (Abbott Laboratories) according to the instructions of the manufacturer. All specimens of each volunteer were analyzed in the same run. The assays are all based on the MEIA technology. The standard of the LH assay is calibrated against the WHO First International Reference Preparation for human LH (68/40), whereas that of the FSH assay is referenced against the WHO Second International Reference Preparation for human FSH (78/549). For progesterone, interassay CV was found to be 14.1% at 3.5 nmol/l; 7.8% at 19.0 nmol/l and 9.9% at 71.2 nmol/l respectively (n=51). For LH between run coefficients of variation were 5.5; 7.2 and 7.9% at 4.8; 39 and 83 IU/l, respectively (n=48). For FSH the between run

CV was 6.0; 6.6 and 8% at levels of 5.0; 25 and 75 IU/l (n=46). Inhibin A and inhibin B levels were measured using an immuno-enzymometric assay (Serotec, Oxford, UK) (Groome *et al.*, 1996). Intra and interassay coefficients of variation for the inhibin A and inhibin B assay were <7.7%; <8.0% and <14.6%; <14.0%, respectively.

*Transvaginal US measurements*

All transvaginal US measurements were performed by the same observer (G.J.S.) using a 7.5-MHz transvaginal probe on a Toshiba Capasee SSA-220A (Toshiba Medical Systems Europe BV, Zoetermeer, The Netherlands). The ovary was examined by scanning from the outer to the inner margin in longitudinal cross-sections, as described by Pache *et al.* (1990). All follicles were measured and counted. Mean follicle diameters were calculated from 2 or 3 perpendicular measurements depending on the diameter (≤6 mm or above) by taking the mean of the measurements. In the analysis of dominant follicles only follicles with a mean diameter ≥10 mm were included because follicle size can vary up to 10 mm before the dominant follicle is identified (Pache *et al.*,1990). Follicles of 2-10 mm in diameter were considered as antral non-dominant follicles. By including follicles up to 10 mm the maximal antral follicle cohort will be represented. Pache *et al.* (1990) found a good accuracy in an in vitro study of small cystic structures as well as a good intra-observer reproducibility of anthral follicle numbers, and sizes of dominant follicles in 7 patients. An inter-observer reproducibility study was performed by Scheffer *et al.* (2000a) in 37 volunteer women, showing an adequate reproducability of small follicle numbers. In earlier studies, O'Herlihy *et al.* (1980) observed a good correlation between the calculated volume of pre-ovulatory follicles and the volume of aspirated follicular fluid. Endometrial thickness was expressed as the total sonographic thickness of the two layers of the endometrium.

*Methods of analysis*

Sonographic and hormonal observations started in the luteal phase (cycle 1), because events in the luteal phase are likely to influence the follicular phase of the subsequent cycle (cycle 2). To synchronize hormonal and ultrasound

data obtained in the course of an ovarian cycle, several time-markers were used. The time after the BBT-rise was used in cycle 1 to describe the subsequent events of the luteal phase. The nadir before a temperature rise was considered as day 0. The start of the menstrual period in the subsequent cycle was used to describe the follicular phase prospectively, and the day of the LH peak (the day the highest LH level during the LH surge was reached) to investigate the follicular phase retrospectively. Finally, the progesterone determinations in this cycle were timed 7 days after ovulation. Ideally, a time-marker is a well-defined, clearly recognizable event, but any of the markers mentioned has its drawbacks. The lack of precision of the BBT-rise as a marker of ovulation is well known (Barrett and Marshall, 1969; Dunson *et al.,* 1999). The time point at which a menstrual period is considered to start, is a subjective choice of the woman, especially when there is no abrupt start of the bleeding as often is the case. It is also uncertain whether the onset of shedding of the endometrium corresponds with the hormonal events, or depends on endometrial factors as well. The LH peak value can be identified by using the highest value of daily results, as we did in the present study. However, the duration and magnitude of the LH surge and thereby the day of the highest value, may vary. In the luteal phase and in the first half of the follicular phase, the volunteers were seen every 2-3 days, which may result in missing values on several days in graphical presentations of data. For that reason, in some parts of the cycles, data of two consecutive days (and in the beginning of the second cycle of cycle days 1-4) were pooled. If results of 2 days were available, only the value of one of these days was used after random selection. In the tables data are given as median values, and 10th. and 90th. percentiles; in the graphs mean values and the 95% interval of the means ($\pm$ 1.96*SEM) are displayed. Statistical analysis was performed by using SPSS (Statistical Package for Social Sciences) for Windows (release 10.0.7). For comparisons of results from different phases of the cycle between age groups the Mann-Whitney U test was applied. A P-value $< 0.05$ (two-sided) and $< 0.01$ were considered statistically significant and highly significant, respectively.

## Results

The group of volunteers consisted of 61 women: 35 in the reference group (22-34 years; median age 31 years); 26 in the group aged 41-46 years (median age 42 years). The body mass index (BMI; body weight/height$^2$) was the same in both groups (median value in both groups 22.7 kg/m$^2$; P = 0.65). There was no statistical difference in smoking habits between the groups. Mean pack-years of smokers and past-smokers in the older group were 9.2 compared to 7.8 in the younger group (P = 0.16). After correction for age, i.e. calculating from the beginning of smoking until the median age of the younger group, results were 5.9 years in the older group and 7.3 years in the younger group, respectively (P = 0.47). Among the 35 younger women the results of hormonal determinations and ultrasound of cycle 2 were incomplete in one woman. This cycle must have been ovulatory because she became pregnant. In the younger group one cycle (2.9%) was judged as abnormal having a cycle length of 16 days and a luteal phase of only 4 days. In the older group 2 abnormal cycles (7.7%) were observed, both showing a persisting follicle. The proportion of abnormal cycles in both groups is not statistically different (P = 0.57). The conception cycle with missing data, and the 3 cycles that were considered as abnormal, were excluded from further analysis. The remaining 33 cycles in the younger age group and 24 cycles in the older age group were considered normal ovulatory. A dominant follicle (>10 mm in diameter) was recognized, which showed a constant growth of approximately 1.6 mm per day, and reached a mean diameter of 15.7 to 26.4 mm before ovulation. In the follicular phases of these cycles, there was a steady increase in $E_2$ concentrations. In one cycle no LH surge was observed. In this cycle of a 42 years old volunteer high levels of FSH and LH were present in the follicular phase. In the luteal phase progesterone levels rose to a normal level of 68 nmol/l. We had no good reason to consider this cycle as abnormal. The data of 33 cycles in the younger and 24 cycles in the older age groups are thus compared in the following sections.

In table I, values of sonographic events and hormonal levels on relevant days of cycles 1 and 2 of both age groups are presented. On the whole, the results in the older group are remarkably similar to those in the reference group of

young women. All women in the elderly group did ovulate, although the mean diameter of the dominant follicle just before ovulation was almost 2 mm smaller as compared to that in the women of the reference group. Some of the differences found were in line with observations from the literature, including the shorter duration of the follicular phase, the elevated early follicular FSH, and a trend to decreased inhibin B levels in the early follicular phase of the older age group. The difference of 2 days at the first appearance of the dominant follicle is in line with the shorter follicular phase in the older women. In the early follicular phase a much lower number of small antral follicles (2-10 mm) was observed in the older group, without a difference in their mean volume. The lower LH peak level in the older group has not been described previously. In both age-groups, inhibin B levels showed an increase after the LH peak. Progesterone levels in the luteal phase and endometrial thickness did not differ at all or were even slightly higher in the elderly group.

**Table I.** Comparison of cycle characteristics, sonographic events and hormonal levels on relevant days of the cycles; median values (10th. - 90th. percentiles)

| Age groups | 22-34 years, n = 33 | 41-46 years, n = 24 | P-value |
|---|---|---|---|
| Total cycle length (days) | 28 (26 - 33) | 26 (23 – 30) | 0.001 |
| Length of follicular phase | 16 (13 - 20) | 12 (10 - 15) | 0.000 |
| Length of luteal phase | 13 (9 - 15) | 14 (12 - 16) | 0.048 |
| Cycle day of first observation of the dominant follicle | 10 (7 - 14) | 8 (5 - 10) | 0.000 |
| Diameter of the dominant follicle just before ovulation (mm) | 21.5 (17.3 - 25.2) | 19,6 (17 - 24.2) | 0.011 |
| Late luteal phase inhibin A (pg/ml) | 9 (2 – 24) | 6.5 (2.6 - 16.4) | 0.15 |
| Late luteal phase inhibin B (pg/ml) | 20 (9 - 97) | 16 (4 - 36.8) | 0.14 |
| Late luteal phase FSH (IU/l) | 4.2 (1.9 - 6.3) | 5.6 (3.4 - 9.6) | 0.002 |
| Highest level of FSH during the late luteal and early follicular phase | 7.5 (5.7 - 12.1) | 9.7 (7.5 - 22.6) | 0.000 |
| Early follicular antral follicle count (AFC) (number) | 10 (5.4 - 25.6) | 4 (2 - 9) | 0.000 |
| Early follicular mean antral follicle volume ($mm^3$) | 45 (23.3 - 89.2) | 51.9 (26 - 122.4) | 0.55 |
| Early follicular $E_2$ (pmol/l) | 172 (100 - 277) | 198 (108 - 337) | 0.32 |
| Early follicular FSH | 6.1 (4.7 - 11.9) | 9.6 (7.4 - 18.1) | 0.000 |
| Early follicular LH (IU/l) | 3.7 (1.6 - 7.3) | 4.9 (2 - 7.3) | 0.09 |
| Early follicular inhibin A | 5 (1.4 - 8.6) | 4 (1.5 - 9.5) | 0.459 |
| Early follicular inhibin B | 87 (30 - 178.2) | 54.5 (10 - 133) | 0.084 |
| Highest level of $E_2$ | 1472 (1113 - 2353) | 1454 (813 - 2033) | 0.26 |
| Highest level of FSH during the LH surge | 14.6 (9 - 19.2) | 14.6 (8.9 - 25.1) | 0.382 |
| Highest level of LH | 59.5 (25.7 - 94.8) | 45.7 (25.1- 74.1) | 0.046 |
| Inhibin B on day of LH peak (pg/ml) | 84 (58.8 - 256.2) | 73 (28 - 127.6) | 0.014 |
| Inhibin B on day after LH peak | 170 (50.6 - 304.4) | 92 (31.2 - 167) | 0.003 |
| Endometrial thickness on day of LH peak (mm) | 8.9 (6.8 - 12.6) | 9.1 (7.6 - 12.1) | 0.966 |
| Endometrial thickness 6-8 days after ovulation | 10.2 (7.8 - 13.8) | 11 (7.6 - 13.5) | 0.345 |
| Progesterone 6-8 days after ovulation (nmol/l) | 50.7 (34.3 - 90.8) | 69.9 (25.8 - 138) | 0.08 |

In Figure 1, several hormone values, and diameters of small antral and dominant follicles are presented in relation to the three time-markers as defined. Results of the luteal phase in the first cycle are displayed in relation to the rise of the BBT, results of the follicular phase in the second cycle are plotted from the start of the menstrual period as well as in retrospect from the day of the LH peak level in order to correct for the observed difference in the length in the follicular phases between the two age-groups.

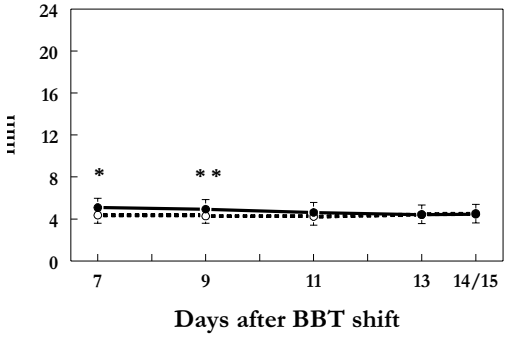
The dominant follicle can be observed from cycle day 8 in the older, and day 10 in the younger age-group. On the corresponding days of the cycle (Figure 1, 1B), the mean follicle diameter in the older group exceeds that of the younger women, and seems to be advanced in the older group by about 2 days relative to the younger group. However, when the diameters are grouped in relation to the LH peak (Figure 1, 1C), the development of the dominant follicle appears to be remarkably similar except for the last 3 days when growth in the older women slows down slightly. As a result, in the older women the dominant follicles are 2 mm smaller, on average, on the day before ovulation (Table I).

The evolution of the $E_2$ levels is in complete agreement with the growth pattern of the dominant follicle (Figure 1, 2B - C).

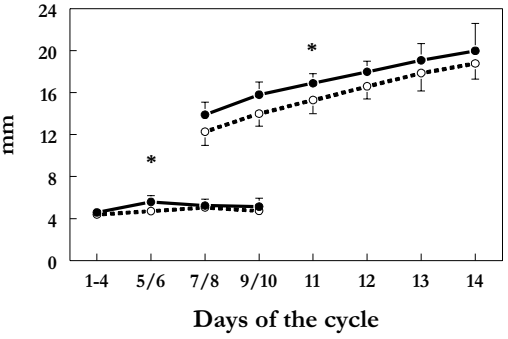
Mean FSH levels in the midluteal phase (7 days after the BBT-shift, Figure 1, 3A) show comparable, low values, rising thereafter both earlier and higher in the older age-group. This earlier rise in FSH is also present on the combined cycle days 1-4, followed by a rise in the younger age group (Figure 1, 3B). In the following days all FSH levels decline, which concurs with the identification and subsequent growth of the leading follicle, and rising $E_2$ levels. The gradual rise after CD 7-8 in the older, and after CD 12 in the younger group is caused by the FSH surge which accompanies the LH surge, that occurs much earlier in the older group. Related to the LH peak level as a time-marker (Figure 1, 3C), the late luteal and early follicular FSH rises can be seen 9-10 days before the day of the LH peak (day 0) in both groups.

LH levels do not differ between the two age-groups except for a lower peak level during the LH surge ($P<0.05$) in the older group.

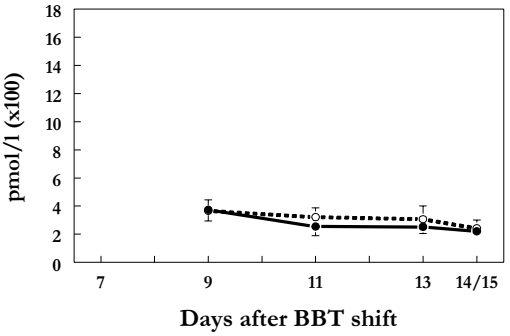
### 1A. Diameter Small Follicles



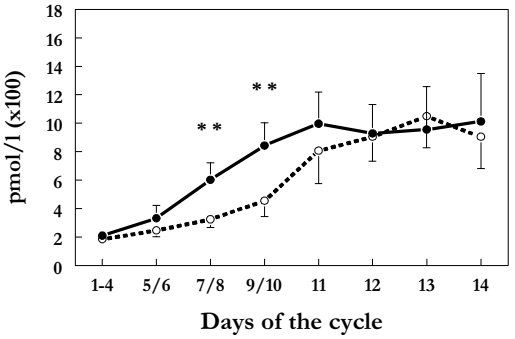

### 1B. Diameter Dominant and Small Follicles



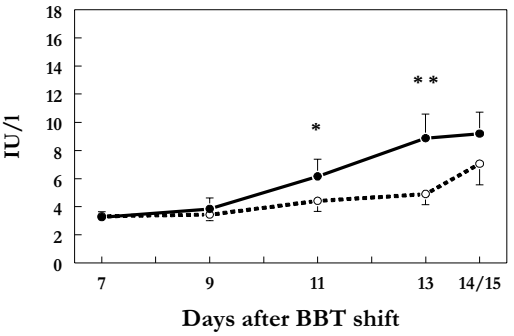

### 2A. Estradiol



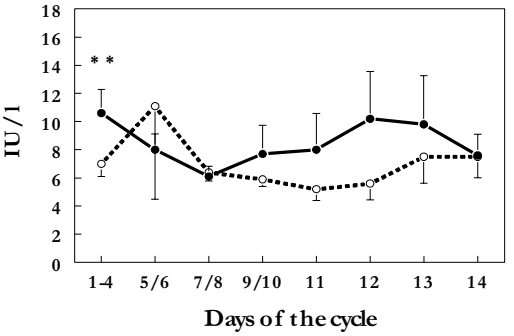

### 2B. Estradiol



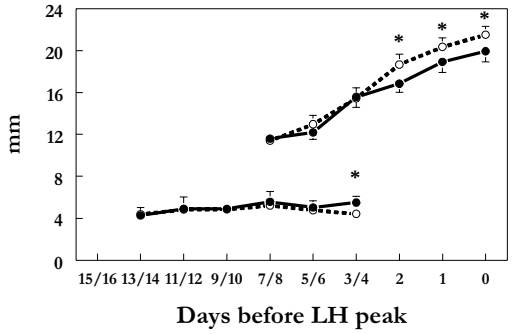

### 3A. FSH



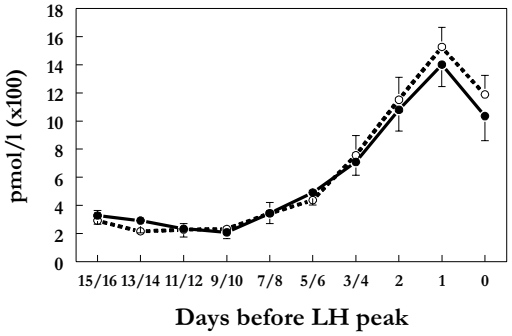

### 3B. FSH



**1C. Diameter Dominant and Small Follicles**




**2C. Estradiol**




**3C. FSH**

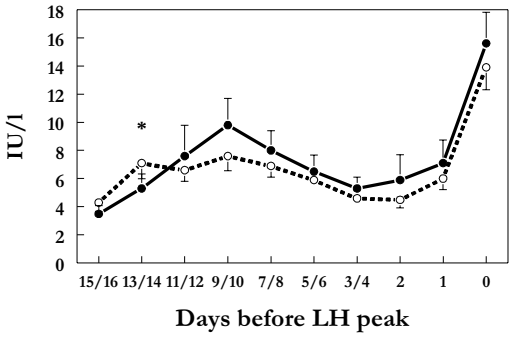



**Figure 1. (1A - 3C).** Small antral (2-10 mm) and dominant follicle diameters (1A, 1B and 1C), estradiol concentrations (2A, 2B and 2C), and FSH concentrations (3A, 3B and 3C), related to the BBT shift in cycle one (panel A), to the start of the menstrual period in cycle two (panel B), and to the LH peak in cycle two (panel C). Data points represent means ± 1.96 SEM.

•••O•••, 22-34 years age group.

———●———, 41-46 years age group.

*, P<0.05.

**, P<0.01.

## Discussion

The first aim of our study was to collect data in a younger age group of fertile women, to be used as a reference for an older group. We considered them to have normal cycles unless definite abnormalities were encountered. In the literature reference groups have been reported, and in some studies criteria for normality have been defined (van Zonneveld *et al.*, 1994). It has been suggested that conception cycles are the gold standard for normality, however, reports on large series are lacking. In addition, also in such 'ideal' cycles, hormone levels and sonographic findings vary greatly. For example, ovulation of a follicle of 13 mm may result in a normal pregnancy (van Zonneveld *et al.*,1994). Moreover, the occurrence of pregnancy is dependent on many female and male factors and it is likely therefore that, on the one hand, many 'ideal' cycles never result in a pregnancy, e.g. because of suboptimal timing of intercourse, whereas, on the other hand, women with suboptimal cycles may conceive, provided all other contributing factors are perfect. The occurrence of pregnancy is the result of a complex and multifactorial process, and it is well possible, therefore, that the achievement of clear-cut and generally applicable criteria of cycle normality is an impossible goal. In the present study we considered the cycles of the younger volunteers as a reasonable reference group because all had earlier succeeded in achieving a spontaneous pregnancy within one year. In this group we found cycles in which the dominant follicle ruptured at smaller diameters (in the younger group 16.3 and 16.7 mm; in the older group 15.7 and 16.7 mm) compared to a diameter reported in the literature as 'normal' (17-25 mm) (O'Herlihy *et al.*,1980; Zegers-Hochschild *et al.*, 1984; Eissa *et al.*, 1986). In some cycles the LH peak level remained relatively low, but criteria for a normal LH surge have never been established. Midluteal progesterone levels not always reached the 'normal' level of 32 nmol/l or more in the younger group (Hull *et al.*,1982, Hamilton *et al.*, 1987; van Zonneveld *et al.*, 1999), and the significance of the progesterone level in cycles that are ovulatory, is unknown.

There is convincing evidence in the literature indicating that the fertility of women above 40 is seriously decreased (Bongaarts, 1982; Wood, 1989; Holman *et al.*, 2000). The second aim of our study was to search for an explanation of this phenomenon. However, our results do not give obvious clues. The

majority of women had seemingly normal, ovulatory cycles with normal estradiol and progesterone levels. In addition the growth curves of the dominant follicle in elderly and younger women until some days before ovulation were very similar. The diameter of the dominant follicle before ovulation was slightly smaller in the older women, but whether this is an indication of diminished follicle quality is unknown.

In the older women the follicular phase of the menstrual cycle was shorter, and in this group the dominant follicle could be identified earlier. Whether the dominant follicle has a faster growth rate in older women ('accelerated growth'), or starts earlier ('advanced growth') (Thatcher and Naftolin, 1991; Klein *et al.*, 1996a) is a matter of debate in the literature. Our data clearly support the latter point of view because the growth pattern of the dominant follicle as well as the rise of estradiol in the follicular phase (see Figure 1, 1B and 1C; Figure 1, 2B and 2C) in older and younger women hardly differ. In case of an 'accelerated' follicle growth, a steeper slope of both the graph of dominant follicle growth and $E_2$ rise would have been expected in the older group. The shift in time of follicle development resulting in a shorter follicular phase in the older age group might be explained by the earlier FSH rise in the luteal phase of the preceding cycle (Figure 1, 3A and 3B). As a result a dominant follicle is selected from the cohort of small recruitable follicles, and stimulated to further growth several days earlier than in the younger group. This concept is shown in Figure 2.

The earlier rise of FSH in the luteal phase of the older women may be related to a declining production of inhibin A by the corpus luteum (Reame *et al.*, 1998; Welt *et al.*,1999; Danforth *et al.*,1998; Santoro *et al.*,1999). We could find a rather strong negative correlation between inhibin A and FSH levels in the late luteal phase (combining both groups, $r = -0.536$; $P = 0.000$), although the difference between older and younger women of inhibin A levels at the late luteal phase, did not reach statistical significance (Table I).

**Figure 2.**

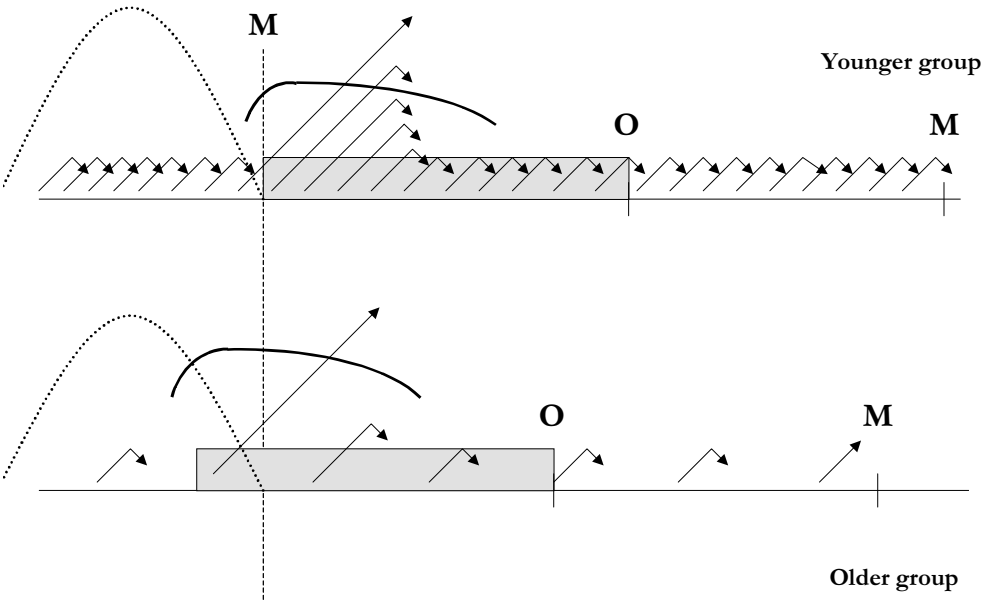



**Figure 2.** Time-relations of progesterone (dotted line), the luteo-follicular FSH rise (bows), the period of follicle growth (grey rectangles), ovulation (O) and onset of menstrual periods (M) in an older (41-46 years) compared to a younger age group (22-34 years). Arrows represent follicular development. Straight arrows indicate growth of a dominant follicle; curved arrows indicate regressing follicles.

Inhibin B levels showed a sharp increase on the day after the LH peak level compared to the previous day (Table I) in the younger (P=0.006) and in the older group (P=0.036). Such a rise has been previously described by Groome *et al.* (1996), and it has been suggested by Speroff *et al.* (1999) that it is a result of release from the ruptured follicle. However, ovulation is not a prerequisite, as a clear rise has also been demonstrated to occur within 24h after the induction of an artificial LH and FSH surge by a single injection of a GnRH-agonist during the early follicular phase (Scheffer *et al.*, 2000b).
An advanced start of a cohort of early antral follicles in older women means

that this start already takes place in the presence of a still functioning corpus luteum (Figure 2). It is possible that the environment for the development of preantral follicles during this phase of the cycle is suboptimal. A second mechanism which may explain the diminished follicle quality in older women is the 'limited oocyte pool model', proposed by Warburton (1989). This hypothesis assumes that the dominant follicle, selected from the cohort of preantral follicles, is the one which benefits longest and most from the favourable effect of FSH during the FSH window. As the number of preantral follicles decreases (in the present study from a mean number of 10 in women below 34 years, to 4 in women between 41-46), it becomes less likely that a follicle will be present at the beginning of the FSH window. Consequently, follicles of older women more often are not yet quite ready or almost too late when selected for further growth in comparison with follicles of younger women (Figure 2). Oocytes of older women, therefore, may be more likely to undergo non-disjunction at the first meiotic division. Moreover, assuming that follicles of different quality appear in the FSH window, it is more likely that a high quality follicle is selected from a cohort of 10 (younger women) than of 4 follicles (older women).

We conclude that in spite of a dramatically decreased number of recruitable antral follicles, follicle development, hormonal events and endometrial growth are remarkably undisturbed in older women. Apparently, a grossly decreased number of antral follicles does not result in cycle disturbances until an average age of 45-46 years. Because an equal endometrial thickness is present in both age groups, a uterine factor seems unlikely. In addition, in oocyte donation the pregnancy rates mainly depend on the age of the donor, not of the recipient (Sauer *et al.*, 1993). However, some decrease in uterine receptivity with age may be present. In recipients over 40 years of age, implantation rates using oocytes of young donors could be enhanced by applying supraphysiological progesterone replacement (Meldrum, 1993). The obvious decrease of fertility in aged women probably has to be mainly explained by a decrease in oocyte quality as is reflected by an increase of chromosomal abberations in oocytes (Brook *et al.*, 1984; Gaulden, 1992; Eichenlaub-Ritter, 1996) and embryos (Warburton *et al.*, 1986; Munne *et al.*, 1994; Holman *et al.*, 2000) of older women. However, as stated above, it can not be excluded that a decrease in oocyte quality may be the result of a decreased quality of the follicle.

There is some evidence that an advanced start of follicle development, as shown in older women, can be manipulated. Le Nestour *et al.* (1993) showed in healthy volunteer women that the intercycle FSH rise can be delayed by administering physiological amounts of estrogens. Hypothetically, in this way in older women the recruitment of a new cohort of follicles, and the selection of a dominant follicle, can be pushed forward from the late luteal phase of the preceding cycle into the early follicular phase. This would result in a situation resembling that in younger women. Whether such an approach would enhance the quality of the cohort of follicles and of the dominant follicle emerging from this cohort, resulting in a more fertile cycle, remains to be confirmed.

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# Chapter 8

GENERAL DISCUSSION, SUMMARY, AND DIRECTIONS FOR FUTURE RESEARCH

# Chapter 8

## General discussion, summary, and directions for future research

This thesis discusses some diagnostic tests in subfertile women with seemingly normal and regular menstrual cycles, and in normal but older women who still have a regular menstrual pattern. The diagnostic tests concern sonographic measures and hormonal patterns reflecting the presence or absence of normal follicle development, ovulation and corpus luteum function in women with a normal and regular cycle pattern.

Anovulatory disorders accompanied with oligo- and amenorrhoea are well known causes of a diminished fertility in women, which can be successfully treated (Hull *et al.*, 1985). Much less is known about subtle disorders of follicle growth, and about hormonal dysregulation in women who have regular menstrual periods. Moreover, little is known about women above 40 who still have regular menstrual cycles, while we know that their fertility is greatly reduced. Cycle regularity implies that the complex hormonal feedback mechanisms, that are governed by follicle growth and the development of a corpus luteum, are intact, and that the quality of such a cycle is expected to be normal.
The general aim of this thesis was to shed some light on this paradox: is it really true that women with regular cycles may have subtle disturbances? How often do they occur and how can we diagnose them? Can they be considered as an explanation of the reduced fertility, or infertility, and how can we use this knowledge for the management of subfertile patients?

There are several reasons why diagnostic tests are performed in medicine, including reproductive medicine (Te Velde *et al.*, 1995).
First, a diagnosis might be important to understand the cause and the mechanism of the disease according to the classical paradigm in medical thinking: only if we know why a patient is ill, we can consider appropriate treatment. In reproductive medicine, however, this principle is more difficult to apply because fertility and infertility are not 'all or nothing' phenomena, like health and disease. Whether or not a woman will conceive within a certain period of time, is determined by the monthly probability of pregnancy, which depends

on numerous, partly unknown, male and female factors. If this probability is zero, a pregnancy will never occur and the couple is truly infertile. However, true infertility is relatively rare (Greenhall and Vessy, 1990). High fertility, normal fertility, subfertility and infertility are to be considered as part of a continuoum, and in many couples there is not one clear-cut cause, but various more or less obvious reasons why a couple has not (yet) conceived, or no obvious reason at all (Te Velde *et al.*, 2000). In that case the subfertility is called 'unexplained'. Nevertheless, it may be important to understand the mechanism also of a relative cause. For example, with regard to the subject of this thesis: it would be important to find out whether or not LUF could be the relative explanation of otherwise unexplained infertility or of reproductive aging. Better understanding of causes and mechanisms is a first condition for designing appropriate therapeutic approaches.
Second, most couples who come to a doctor with the complaint of infertility, are either subfertile or even have a normal fertility. Only a small proportion of them is truly infertile. It is important to be able to distinguish between these possibilities and recognize the couples who would benefit from immediate treatment and those who can better wait some time and try to conceive spontaneously (prognostic aim).
Third, if treatment is to be considered, diagnostic tests are important to decide which modality of treatment is most appropriate for this particular couple (therapeutic aim).

In this era of scientific progress, it is often assumed that clinical decisions are firmly based on results of research. The movement of Evidence-Based Medicine (EBM) teaches us, however, that most clinical decisions have no scientifically proven base. Only with regard to decide whether or not certain modalities of treatment are useful and effective, considerable progress has been made by analysing the results of pooled randomized controlled trials (meta-analyses). Also in reproductive medicine EBM has made some progress, although most treatments which are applied at present, are not (yet) evidence-based. Because of the multivariate nature of reproductive medicine, almost no progress has been made in EBM of diagnostic tests, both with regard to the prognostic aim and to the therapeutic aim. This statement is sometimes interpreted as if diagnostic tests in reproductive medicine are more or less worthless.

In Table 1, it is attempted to distinguish various levels of knowledge on which clinical decisions are based, varying from no knowledge at all to absolute certainty. We have to conclude, that most of the decisions based on diagnostic tests in reproductive medicine, have not surpassed the degree of knowledge present in level 4. As long as the level of evidence-based medicine has not been reached, we have to base our clinical decisions on the level of knowledge present in level 4. The latter two statements also apply for the diagnostic tests which are studied in this thesis.

**Table 1.** Levels of knowledge on which clinical decisions are based*

| | |
|---|---|
| 1. | No knowledge at all. |
| 2. | Speculative knowledge, based on theories and religious notions. Often authority-based. |
| 3. | Biological knowledge, based on laboratory experiments and animal research. |
| 4. | Knowledge based on non-controlled trials, common sense, intuition and experience. Biological plausibility (level 3) is also integrated in this level. |
| 5. | Evidence-based knowledge based on the results of randomized controlled trials. |
| 6. | Knowledge which is based on absolute certainty. |

* adapted from Te Velde, 1996

With regard to definitions of LUF and other subtle ovulation disorders, we have to conclude that the confusion is great (Chapter 1). Follicles of normal pre-ovulatory size that do not rupture after the LH surge, may either continue to grow (these cycles are called 'cyst cycles' by some authors and 'LUF cycles' by others), or diminish in size, which cycles are generally called 'LUF cycles'. Cycles in which follicles do not reach a diameter larger than between 16 and

17 mm, generally are called 'poor follicle growth cycles', but 'asynchronous cycles' by others. In some publications it remains unclear, whether or not such small follicles ovulated. We propose to reserve the term LUF for those conditions where, in spite of the fact that the follicle does not rupture, luteinization with progesterone production occurs and normal cycle regularity is maintained. We think that in comparison with LUF the clinical relevance of follicles that ovulate at a too small diameter, is still speculative.

The most important conclusions from the literature with regard to LUF (Chapter 1) are the following.

a. In comparison with subfertile patients LUF is rare in cycles of normal fertile women (Chapter 1, Tables 5 and 7). The results indicate that LUF is likely to be a relative cause of subfertility, specially if this is unexplained (Chapter 1, Table 5).
   The recurrence rate is not yet well established (Chapter 1, Table 6), but it is obvious that LUF cycles can be followed by ovulatory cycles.
b. Whether the presence of LUF is relevant for the prognosis, is completely unknown.
c. With regard to the therapeutic aim, the results of Table 8 (Chapter 1) suggest that ovulation induction with gonadotrophins might be beneficial. This suggestion is based on some observational studies (level 4) and needs to be confirmed in RCT's. For the time being, an attempt to treat LUF with gonadotrophins seems warranted.

The first aim of this thesis was to investigate how frequent disorders of follicle growth, ovulation and hormonal patterns can be diagnosed in subfertile women with regular cycles and low serum progesterone levels during the luteal phase of previous cycles. We performed serial hormonal and ultrasound measurements in a group of 50 women who had relatively low serum progesterone levels (< 32 nmol/l) in two cycles. According to minimal criteria from the literature to consider a cycle as normal, 25 cycles showed abnormalities. Fourteen cycles were classified as LUF cycles. In eight cycles, follicles remained too small, in 2 cycles no follicle was observed during the LH surge, and in 1 cycle there was no follicle development. In 16 other cycles progesterone levels were again relatively low, but because in these cycles normal follicle growth

and ovulation occurred, it seems not justified to consider such cycles as abnormal. However, it can not be excluded that the ability to conceive during those cycles is reduced (Hull *et al.*, 1982). In a sample of 171 women from the total patient population, almost 9% had two low-progesterone cycles. This would suggest that in 2-3% of the initial patients a LUF cycle was present. However, Hamilton *et al.* (1985) found that half of their LUF patients had progesterone levels < 32 nmol/l, the other half had levels > 32 nmol/l. If we extrapolate this figure, this would mean that we missed half of the patients with LUF cycles. Thus, the real incidence of LUF cycles was estimated at about 5%. We also concluded that low progesterone levels predict subtle cycle disorders in following cycles. An incidence of about 5% is lower than has been described in most publications. However, in control groups of normal fertile women, subtle cycle disorders are rarely seen. In our two age-groups of volunteers (22-34 years and 41-46 years, including 61 women in total) who all had succeeded to become pregnant within one year in the past (Chapter 7), three cycles were abnormal. None of these cycles were, however, abnormal in the sense of a LUF cycle. One cycle was too short, the two other cycles showed persisting follicles, which means that no luteinization occurred. We concluded that LUF cycles probably occur more often in a subfertile than in a proven fertile population. In patient groups, *recurrence* of subtle cycle disorders, however, with quite some differences in the incidence, have been found. We could predict the occurrence of subtle disorders in subsequent cycles. In our opinion this is an argument in favour of the assumption that subtle cycle disorders recur in at least a part of the patients.

The second aim of the thesis was to estimate the cost-effectiveness of different diagnostic procedures to diagnose subtle cycle disorders. The costs were related to the possible benefits, from literature data. We calculated the costs for diagnosing such disorders by a 'maximal diagnostic' strategy applying vaginal ultrasound examinations combined with hormone estimates in all women with regular cycles; by an 'ultrasound-only' approach in all women; and by a 'preselection' strategy with progesterone measurements in all women, followed by ultrasound and hormone determinations in women who had progesterone levels < 32 nmol/l in two cycles. If only *medical* costs were considered, diagnosis of one patient with a subtle cycle disorder using the 'maximal' strategy required a total medical cost of 9057 european currency units (Euro's),

by 'ultrasound-only' ECU 4520 and by 'preselection' ECU 3036. However, especially by applying the latter method in a considerable number of cases the diagnosis may be missed. In several textbooks and articles luteal phase progesterone measurements in the work-up of subfertile couples are recommended (Chapter 5), but further diagnostic steps are not mentioned. Based on the results of our study we conclude that the 'ultrasound-only' approach is the most cost-effective.

Therapeutic advices include the use of various drugs for ovulation induction treatment. Results of these treatments, reported in the literature, are promising (Chapter 1, Table 8).

We conclude that it may be useful to search for subtle ovulation disorders in the work-up of regularly cycling subfertile women. Research on providing knowledge on level 5 (Table 1) with regard to the prognostic aim, would require to investigate many women with LUF cycles and compare them to women with ovulatory cycles, but otherwise the same characteristics, and follow them for several cycles in order to be able to conclude whether the presence of LUF is a poor prognostic sign. With regard to the therapeutic aim, a large group in which LUF was found, should be treated and (randomly) compared with women with LUF without treatment. It probably is difficult to motivate a sufficient number of patients to participate in such demanding, multicentered, studies. In this era of in vitro fertilization (IVF), patients can be offered a treatment that could be expected to bypass several cycle anomalies, but to the best of our knowledge this has not been investigated. IVF treatments are emotionally and financially demanding and have several risks, including multiple pregnancies, the ovarian hyperstimulation syndrome, bleeding and infection.

The third aim of the thesis was to investigate whether ovulation disorders or abnormal hormonal patterns occur frequently in women with oligo- or amenorrhoea who receive a symptomatic treatment (ovulation induction) with the estrogen receptor blocking agent clomiphene citrate (CC). We compared the first treatment cycles of 24 patients with cycles of 27 age-matched control women who had proven their natural fertility in the past. In 11 CC-stimulated cycles no follicle development whatsoever occurred. One cycle showed the features of a LUF cycle and the remaining 12 cycles were ovulatory. We concluded that an increased rate of subtle cycle disorders is not an explanation for

a discrepancy between assumed ovulation rate and pregnancy rate with CC. A further observation in this study was that in 4 of 10 hormonally evaluable patients a temporary decline of estradiol levels was present between cycle days 7 and 11, compared to 1 woman in the control group. It appeared that in those cycles the estradiol levels reached higher values on cycle day 7 or 8 than in cycles not showing an estradiol-decline. Higher estradiol levels appeared to be positively related to age, and we hypothesized that follicles of the older women show a diminished production of inhibin in response to ovarian stimulation. As a consequence the negative feedback towards the central system is subnormal in the older patients, resulting in higher follicle-stimulating hormone (FSH) levels (Hughes *et al.*, 1990). It is unknown, whether cycles showing high and temporally declining estradiol levels are less fertile. To investigate this, many cycles should be analyzed. If such cycles would appear to be less fertile, another therapy could be chosen, thus saving the patients' time and efforts. A matter of interest is, whether such patterns of estradiol can be used as a diagnostic test for ovarian aging. At present a clomiphene challenge test is in use, in which, after administering 100 mg of clomiphene citrate daily during cycle day 5-9, an FSH level on cycle day 10 that exceeds a certain threshold is used as an index of ovarian competence. The value of this test, however, has been challenged (Bancsi *et al.*, 2001). Possibly, adding the estradiol-response on cycle day 7 or 8 may improve the predictive value of this test. Licciardi *et al.* (1995) found that relatively high estradiol levels on cycle day 3 predicted low oocyte yields and low pregnancy rates after IVF in the same cycles. Evers *et al.* (1998) showed that normal early follicular FSH levels, in combination with high estradiol levels, in a preceding cycle, predicted a poor response to stimulation for IVF. In an alternative CC-challenge test, as proposed above, it may be advantageous to combine the estradiol levels on cycle day 3 (before CC) and cycle day 7-8.

The fourth aim of the thesis was to obtain reference values for cycle normality from a group of relatively young fertile women (22-34 years) with regular menstrual cycles, and to evaluate whether follicle growth, ovulation, endometrial development and hormonal patterns in older women (41-46 years) differ from the reference group, and, if present, whether such differences may explain, or contribute to, the age-related loss of fertility. The women in both groups had became pregnant within a year in the past. The older group in-

cluded women with an age-range in which fertility can be expected to be greatly impaired. The younger women were in an age-range in which fertility is optimal or only slightly diminished. The size and growth of follicles were followed by vaginal ultrasound, and levels of several hormones were measured during the cycle. It appeared that clearly abnormal cycles were rare: in the older group 2, compared to 1 in the younger women. This means that cycle disorders such as a LUF and other subtle cycle disorders do not explain the age-related decline of fertility. Some differences were found between the age groups. The total cycle length was shorter in the older women, which was caused by a shorter follicular phase. Until now, such a shorter follicular phase is thought to result from a faster growth of follicles, starting in the early follicular phase ('accelerated' follicle development). Our results indicate, however, that there is no difference in the time-span in which follicles are recruited and stimulated to further growth, the dominant follicle is selected, and ovulation takes place. The difference between older and younger women is a *shift in time* of 2-3 days of follicle development and estradiol increase which already starts during the late luteal phase of the preceding cycle. Our results clearly demonstrate that there is an 'advanced' follicle development instead of an 'accelerated' one. The earlier rise of FSH levels in the older group, starting already during the luteal phase of the preceding cycle, could well be the trigger for earlier follicle growth and further development. We hypothesize that the earlier rise of FSH is caused by a diminished production of inhibin A by the corpus luteum of the older women (Danforth *et al.*, 1998, Santoro *et al.*, 1999). Inhibin is known to lower selectively the pituitary FSH production, by a negative feedback mechanism. Estradiol and progesterone both have a negative feedback on FSH (and LH), but estradiol and progesterone levels did not differ during the luteal phase between both groups. The fall of progesterone, at the end of the life-span of the corpus luteum, is responsible for the onset of the menstrual period (Le Nestour *et al.*, 1993). Therefore, the earlier rise of FSH can advance follicle development without advancing menstruation. Another difference between the age groups was a decreasing growth rate of the dominant follicle during the 3 days preceding ovulation in the older women, resulting in a smaller diameter before ovulation. Estradiol levels and endometrial thickness did not differ in this stage of the cycle. It is generally believed that the decline of fertility with age results from a decline in oocyte quality, by

an increasing incidence of chromosomal aberrations. However, less favourable conditions during follicle development may contribute to this. The earlier start, during the preceding luteal phase, may occur during a suboptimal hormonal environment. The smaller follicle diameter before ovulation may be a reflection of such a less favourable start. Other reasons why follicle quality (and consequently oocyte quality) may be diminished is the lower number of recruitable follicles (due to a diminished resting follicle pool) during the 'FSH-window' which may lead to an exposure to FSH of these follicles that lasts too short for optimal further development (Warburton, 1989). In addition, selection of a dominant follicle from a smaller cohort may, just by chance, results in a follicle of less quality. It is tempting to speculate that the advanced follicle development in older women can be manipulated. It has been shown by Le Nestour *et al.* (1993) that the early follicular FSH rise can be postponed by the administration of physiological amounts of estrogens. Advantages of such a procedure could be a better timing of the postcoital test (De Ziegler *et al.*, 1999) or better timing of intra-uterine insemination (De Ziegler *et al.*, 1998; 1999). Another suggestion is that at the start of a hyperstimulated cycle for IVF endogenous and exogenous FSH could be better synchronized (De Ziegler *et al.*, 1998). It might also be possible to postpone the earlier FSH rise in older women from the late luteal phase of the preceding cycle towards the early follicular phase, the normal situation in younger women. Whether follicle growth and hormonal events would resemble more those of younger women, and whether this results in a more fertile cycle, could be the subject of further research.

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# Hoofdstuk 9

ALGEMENE DISCUSSIE, SAMENVATTING, EN AANWIJZINGEN VOOR TOEKOMSTIG ONDERZOEK

# Hoofdstuk 9

## Algemene discussie, samenvatting, en aanwijzingen voor toekomstig onderzoek

In dit proefschrift worden enkele diagnostische tests ter discussie gesteld, die worden uitgevoerd bij subfertiele vrouwen met ogenschijnlijk normale en regelmatige cycli, en bij normale maar oudere vrouwen die nog een regelmatig menstruatiepatroon hebben. De diagnostische tests betreffen echoscopische metingen en hormonale patronen die de aan- of afwezigheid van een normale ontwikkeling van follikels, ovulatie, en functie van het corpus luteum aangeven, bij vrouwen met een normaal en regelmatig patroon van hun cyclus.

Anovulatoire stoornissen die gepaard gaan met oligo- en amenorroe, zijn bekende oorzaken van een verminderde vruchtbaarheid van vrouwen, die met goed resultaat kunnen worden behandeld (Hull *et al.*, 1985). Er is veel minder bekend over subtiele stoornissen van de follikelgroei, en over hormonale ontregeling bij vrouwen die regelmatige menstruele cycli hebben. Bovendien is er weinig bekend over vrouwen die ouder zijn dan 40 jaar en nog regelmatig menstrueren, terwijl wij weten dat hun vruchtbaarheid sterk is afgenomen. Een regelmatige cyclus betekent dat de ingewikkelde hormonale terugkoppelingsmechanismen, die beheerst worden door de groei van follikels en de ontwikkeling van een corpus luteum, intact zijn, en dat verwacht kan worden dat de kwaliteit van zo'n follikel normaal is.
De algemene doelstelling van dit proefschrift was deze tegenstelling te belichten: is het werkelijk waar dat vrouwen met een regelmatige cyclus subtiele verstoringen van hun cyclus kunnen hebben? Hoe vaak komen deze voor en hoe kunnen wij de diagnose stellen? Kunnen zij beschouwd worden als een verklaring voor de afgenomen vruchtbaarheid, of de onvruchtbaarheid, en hoe kunnen we deze kennis gebruiken voor het beleid bij verminderd vruchtbare patiënten?

Er zijn verscheidene redenen waarom diagnostische tests worden uitgevoerd in de geneeskunde, waaronder de voortplantingsgeneeskunde

(Te Velde *et al.*, 1995).
In de eerste plaats kan een diagnose belangrijk zijn om de oorzaak en het mechanisme van de ziekte te begrijpen, in overeenstemming met het klassieke medisch-wetenschappelijke uitgangspunt: alleen als we weten waardoor een patiënt ziek is, kunnen we een goede behandeling bedenken. In de voortplantingsgeneeskunde is het echter moeilijker dit principe toe te passen, omdat vruchtbaarheid en onvruchtbaarheid geen 'alles of niets' verschijnselen zijn zoals gezondheid en ziekte. Of een vrouw al of niet zwanger zal worden binnen een zeker tijdsinterval, wordt bepaald door de maandelijkse zwangerschapskans, die afhankelijk is van talrijke, gedeeltelijk onbekende, mannelijke en vrouwelijke factoren. Als deze kans nul is, zal een zwangerschap nooit optreden, en in dat geval is het paar werkelijk onvruchtbaar. Echter, echte onvruchtbaarheid is relatief zeldzaam (Greenhall and Vessy, 1990). Hoge vruchtbaarheid, normale vruchtbaarheid, verminderde vruchtbaarheid en onvruchtbaarheid kunnen worden beschouwd als onderdelen van een glijdende schaal, en bij veel paren is er niet één duidelijke oorzaak, maar zijn er verschillende, meer of minder duidelijke oorzaken waardoor een paar (nog) geen zwangerschap heeft bereikt, of is er in het geheel geen duidelijke oorzaak (Te Velde *et al.*, 2000). In dat geval wordt de vruchtbaarheidsstoornis 'onverklaard' genoemd. Niettemin kan het belangrijk zijn om ook het werkingsmechanisme van een relatieve oorzaak te begrijpen. Bijvoorbeeld, met betrekking tot dit proefschrift: het zou van belang zijn te ontdekken of (of 'luteinized unruptured follicle' (LUF) cycli al of niet de relatieve verklaring kunnen zijn van een overigens onverklaarde vruchtbaarheidsstoornis, of van de leeftijdsafhankelijke afname van de vruchtbaarheid. Een beter begrip van de oorzaken en de mechanismen is een eerste voorwaarde voor het ontwerpen van een doeltreffende behandeling.
In de tweede plaats zijn de meeste paren die naar een arts gaan met als klacht dat er nog geen zwangerschap is ontstaan, óf slechts verminderd vruchtbaar, óf zelfs normaal vruchtbaar. Slechts een klein deel van hen is werkelijk onvruchtbaar. Het is belangrijk, onderscheid te kunnen maken tussen deze mogelijkheden, en de paren te herkennen die zouden profiteren van een onmiddellijke behandeling, evenals de paren die beter enige tijd kunnen wachten om te proberen spontaan een zwangerschap tot stand te brengen (prognostische doelstelling).

In de derde plaats zijn, als een behandeling wordt overwogen, diagnostische tests belangrijk om te beslissen welke behandelwijze het meest bij dit afzonderlijke patiëntenpaar past (therapeutische doelstelling).

In dit tijdperk van wetenschappelijke vooruitgang wordt vaak aangenomen dat klinische beslissingen stevig zijn gebaseerd op de resultaten van wetenschappelijk onderzoek. De movement of Evidence-Based Medicine (EBM, dit is een proces van systematisch opzoeken, beoordelen en samenvatten van bij voorkeur gerandomiseerde studies) leert ons echter dat de meeste klinische beslissingen geen wetenschappelijk bewezen basis hebben. Uitsluitend om te kunnen uitmaken of bepaalde behandelmethoden nuttig en effectief zijn, is er aanzienlijke vooruitgang geboekt door het analyseren van de resultaten van gepoolde gecontroleerde onderzoeken (meta-analyses). Ook in de voortplantingsgeneeskunde heeft EBM enige vooruitgang geboekt, hoewel de meeste vormen van behandeling die worden toegepast, (nog) niet 'evidence-based' zijn. Doordat in de voortplantingsgeneeskunde vele factoren een bijdrage leveren, is er bijna geen vooruitgang geboekt in de EBM van diagnostische tests, zowel wat betreft de prognostische als de therapeutische doelstelling. Deze vaststelling wordt soms uitgelegd alsof diagnostische tests in de voortplantingsgeneeskunde in meerdere of mindere mate zonder waarde zijn. In Tabel 1 wordt getracht verschillende niveaus van kennis te onderscheiden waarop klinische beslissingen zijn gebaseerd, variërend van totaal geen kennis tot absolute zekerheid. We moeten concluderen dat in de voortplantingsgeneeskunde de meeste beslissingen die gebaseerd zijn op diagnostische tests, niet uitkomen boven het kennisniveau dat overeenkomt met niveau 4. Zolang het niveau van de evidence-based medicine niet is bereikt, moeten we onze klinische beslissingen baseren op de kennis van niveau 4. De laatste twee opmerkingen zijn ook van toepassing op de diagnostische tests die in dit proefschrift het onderwerp van studie zijn.

**Tabel 1.** Niveaus van kennis waarop het klinische handelen is gebaseerd*

| | |
|---|---|
| 1. | Geen enkele kennis. |
| 2. | Speculatieve kennis gebaseerd op theoretische en religieuze overwegingen. Dikwijls gebaseerd op autoriteit. |
| 3. | Biologische kennis op grond van laboratoriumbevindingen of dierexperimenten. |
| 4. | Kennis gebaseerd op niet-gecontroleerde studies, gezond verstand, intuïtie en ervaring. Biologische plausibiliteit (niveau 3) behoort ooktot dit niveau. |
| 5. | 'Evidence-based': kennis op basis van de resultaten van gerandomiseerd gecontroleerd klinisch onderzoek. |
| 6. | Kennis gebaseerd op absolute zekerheid. |

* naar Te Velde, 1996

Wat betreft de definitie van LUF en andere subtiele ovulatiestoornissen, moeten we vaststellen dat de verwarring groot is (Hoofdstuk 1). Follikels met normale pre-ovulatoire afmetingen die niet springen na de LH piek, kunnen óf doorgroeien (deze cycli worden door sommige auteurs 'cyste-cycli' genoemd, en 'LUF cycli' door andere), óf kleiner worden, welke cycli in het algemeen 'LUF cycli' worden genoemd. Cycli waarin de follikels niet een doorsnede bereiken die groter is dan tussen 16 en 17 mm, worden in het algemeen 'cycli met slecht groeiende follikels' genoemd, maar door anderen 'asynchrone cycli'. In sommige publikaties blijft het onduidelijk of zulke kleine follikels al of niet ovuleerden. Wij stellen voor de term LUF te reserveren voor die omstandigheden waarin, ondanks het feit dat de follikel niet springt, luteïnisering gepaard gaande met de productie van progesteron optreedt, en de normale regelmatigheid van de cyclus blijft bestaan. Wij denken, dat in vergelijking met LUF het klinische belang van follikels die ovuleren bij een te kleine diameter, nog geheel speculatief is.

De belangrijkste conclusies uit de literatuur met betrekking tot LUF (Hoofdstuk 1) zijn de volgende:

a. In vergelijking tot subfertiele patiënten is LUF zeldzaam in cycli van normaal vruchtbare vrouwen (Hoofdsuk 1, Tabel 5 en 7). De resultaten maken waarschijnlijk dat LUF een relatieve oorzaak is van verminderde vruchtbaarheid, in het bijzonder als deze onverklaard is (Hoofdstuk 1, Tabel 5).

   De mate van herhaling is nog niet goed vastgesteld (Hoofdstuk 1, Tabel 6), maar het is duidelijk dat LUF cycli kunnen worden gevolgd door ovulatoire cycli.

b. Of de aanwezigheid van LUF cycli van belang is voor de prognose, is geheel onbekend.

c. Met betrekking tot de therapeutische doelstelling, suggereren de resultaten in Tabel 8 (Hoofdstuk 1) dat ovulatie-inductie met gonadotrofinen van voordeel zou kunnen zijn. Dit is gebaseerd op enkele waarnemings-onderzoeken (kennisniveau 4), maar dit moet nog worden bevestigd met gerandomiseerd onderzoek, in vergelijking met een controlegroep. Vooralsnog lijkt een poging om LUF met gonadotrofinen te behandelen gerechtvaardigd.

De eerste doelstelling van dit proefschrift was te onderzoeken hoe frequent stoornissen in de follikelgroei, de ovulatie en de hormonale patronen kunnen worden vastgesteld bij subfertiele vrouwen met een regelmatige cyclus en lage progesteronwaarden in het bloedserum tijdens de luteale fase van voorafgaande cycli. Wij deden opeenvolgende hormoonbepalingen en echoscopische metingen bij een groep van 50 vrouwen die relatief lage serum-progesteronwaarden (< 32 nmol/l) hadden in 2 cycli. Volgens minimale criteria vanuit de literatuur, om een cyclus als normaal te mogen beschouwen, toonden 25 cycli afwijkingen. Veertien cycli werden geklassificeerd als LUF cycli. In 8 cycli bleven de follikels te klein, in 2 cycli werd geen follikel waargenomen tijdens de LH piek, en in 1 cyclus was er geen ontwikkeling van een follikel. In 16 andere cycli waren de progesteronwaarden opnieuw relatief laag, maar aangezien in deze cycli een normale follikelgroei en ovulatie optraden, lijkt het niet gerechtvaardigd deze cycli als abnormaal te beschouwen. Het kan echter niet worden uitgesloten dat de mogelijkheid om in deze cycli zwanger

te worden, is verminderd (Hull *et al.*, 1982). In een steekproef van 171 vrouwen uit de totale patiëntenpopulatie had bijna 9% van hen 2 cycli met lage progesteronwaarden. Dit zou kunnen betekenen dat bij 2-3% van de oorspronkelijke patiënten een LUF cyclus aanwezig was. Echter, Hamilton *et al.* (1985) vonden dat de helft van hun LUF patiënten progesteronwaarden < 32 nmol/l hadden, de andere helft > 32 nmol/l. Als we dit getal extrapoleren, zou dat betekenen dat wij de helft van de patiënten met LUF cycli zouden hebben gemist. Daarom werd de werkelijke incidentie van LUF cycli geschat op ongeveer 5%. Wij concludeerden ook, dat lage progesteronwaarden een voorspellende waarde hebben voor het optreden van subtiele stoornissen in een volgende cyclus.

Een incidentie van ongeveer 5% is lager dan in de meeste publicaties is beschreven. Echter, bij controlegroepen van normaal vruchtbare vrouwen worden subtiele cyclusstoornissen zelden gezien. In onze twee leeftijdsgroepen van vrijwilligsters (22-34 jaar en 41-46 jaar, totaal 61 vrouwen), die er allen in het verleden in waren geslaagd binnen een jaar zwanger te worden (Hoofdstuk 7), waren 3 cycli afwijkend. Geen van deze cycli was echter afwijkend in de zin van een LUF cyclus. Eén cyclus was te kort, de 2 andere cycli toonden een persisterende follikel, hetgeen betekent dat er geen luteïnisatie optrad. Wij concludeerden dat LUF cycli waarschijnlijk vaker voorkomen in een verminderd vruchtbare dan in een bewezen vruchtbare populatie. In patiëntengroepen is *herhaling*, echter met een nogal verschillende incidentie, gevonden. Wij bleken het optreden van subtiele afwijkingen in een volgende cyclus te kunnen voorspellen. Naar onze mening is dit een argument voor de veronderstelling dat subtiele cyclusstoornissen zich herhalen bij ten minste een deel van de patiënten.

De tweede doelstelling van het proefschrift was het bepalen van de kosten-effectiviteit van verschillende diagnostische procedures om subtiele cyclusstoornissen vast te stellen. De kosten werden gerelateerd aan de mogelijke voordelen voor de patiënten, op basis van literatuurgegevens. Wij berekenden de kosten voor het stellen van de diagnose met een 'maximaal diagnostische' strategie waarbij vaginaal echoscopisch onderzoek werd gebruikt, in combinatie met hormoonbepalingen bij alle vrouwen met een regelmatige cyclus; een 'uitsluitend echoscopie' strategie bij alle vrouwen; en een 'voorselectie' strategie met progesteronbepalingen bij alle vrouwen, gevolgd door echoscopie en

hormoonbepalingen bij vrouwen die in 2 cycli uitsluitend progesteronwaarden < 32 nmol/l hadden. Indien alleen *medische* kosten werden beschouwd, vereiste de diagnose van een subtiele cyclusstoornis per patënte met behulp van de 'maximale' strategie een bedrag van 9.057 euro aan totale medische kosten, de 'uitsluitend echo' strategie 4.520 euro, en de 'voorselectie' strategie 3.036 euro. Bij het toepassen van de laatstgenoemde methode kan echter in een aanzienlijk aantal gevallen de diagnose worden gemist. In verscheidene leerboeken en artikelen worden progesteronbepalingen bij de diagnostiek van subfertiele paren aanbevolen (Hoofdstuk 5), maar verdere diagnostische stappen worden hierbij niet genoemd. Op basis van de resultaten van ons onderzoek concluderen wij dat de 'uitsluitend echoscopie' aanpak de meest kosten-effectieve is.
Behandeladviezen betreffen de toepassing van diverse medicamenten voor ovulatie-inductie behandeling. De resultaten hiervan, zoals die zijn gepubliceerd, zijn veelbelovend (Hoofdstuk 1, Tabel 8).
Wij trokken de conclusie, dat het nuttig kan zijn bij de diagnostiek van subfertiele vrouwen met een regelmatige cyclus subtiele ovulatiestoornissen op te sporen. Research met het doel kennis te vergaren op niveau 5 (Tabel 1) om te beantwoorden aan de prognostische doelstelling, zou vereisen dat veel vrouwen met LUF onderzocht moeten worden, en worden vergeleken met vrouwen die een ovulatoire cyclus, maar overigens dezelfde kenmerken hebben. Zij zouden gedurende meerdere cycli vervolgd moeten worden om te kunnen concluderen of de aanwezigheid van LUF cycli een prognostisch ongunstig teken is. Om aan de therapeutische doelstelling te kunnen beantwoorden, zou een grote groep vrouwen bij wie LUF cycli werden gevonden, at random moeten worden verdeeld in een groep die wel, en een groep die niet wordt behandeld. Het is waarschijnlijk moeilijk een voldoende groot aantal vrouwen te motiveren om aan zodanig veeleisende multicenter onderzoeken deel te nemen. In dit tijdperk van in vitro fertilisatie (IVF) kan de patiënten een behandeling worden geboden, waarvan te verwachten valt dat deze verscheidene cyclusanomalieën kan omzeilen, maar voor zover ons bekend, is dit nooit onderzocht. IVF behandelingen zijn in emotioneel en financieel opzicht belastend en brengen diverse risico's met zich mee, zoals meerlingzwangerschappen, het ovarieel hyperstimulatiesyndroom, bloeding en infectie.
De derde doelstelling van het proefschrift was te onderzoeken of ovulatiestoornissen of afwijkende hormonale patronen frequent voorkomen

bij vrouwen met oligo- of amenorroe, die hiervoor een symptomatische behandeling ontvangen (ovulatie-inductie) met de oestrogeen receptor blokkerende stof clomifeen citraat (CC). Wij vergeleken de eerste behandelcyclus van 24 patiënten met cycli van 27 controlevrouwen van dezelfde leeftijdsgroep, die hun natuurlijke vruchtbaarheid eerder hadden bewezen. In 11 cycli die met CC werden gestimuleerd, trad in het geheel geen ontwikkeling van een follikel op. Eén cyclus toonde de kenmerken van een LUF cyclus, en de overige 12 cycli waren ovulatoir. Wij concludeerden hieruit, dat een toegenomen mate van voorkomen van subtiele cyclusstoornissen geen verklaring vormt voor een discrepantie tussen het de percentages ovulaties per cyclus en zwangerschappen per cyclus. Een verdere waarneming in deze studie was dat bij 4 van 10, wat hormoonbepalingen betreft goed te evalueren patiënten, een tijdelijke daling de oestrogeenconcentraties aanwezig was tussen cyclusdag 7 en 11, in vergelijking tot 1 vrouw in de controlegroep. Het bleek dat in deze cycli de bloedspiegels van oestradiol hogere waarden bereikten op cyclusdag 7 of 8 dan in cycli die geen oestradioldaling vertoonden. Verder bleek dat hogere oestradiolwaarden positief correleerden met de leeftijd. Wij stelden de hypothese op, dat follikels van oudere vrouwen een verminderde productie van inhibine tonen in respons op stimulatie van de ovaria. Als gevolg hiervan, is de negatieve terugkoppeling naar het centrale systeem bij de oudere patiënten subnormaal, hetgeen leidt tot hogere spiegels van het follikel stimulerend hormoon (FSH) (Hughes *et al.*, 1990). Het is onbekend of in cycli waarin hoge, en tijdelijk dalende, oestradiolspiegels voorkomen, de vruchtbaarheid lager is. Om dit te onderzoeken zouden vele cycli nodig zijn. Als in zulke cycli de vruchtbaarheid verminderd zou blijken te zijn, zou voor een andere behandeling kunnen worden gekozen, hetgeen de patiënte tijd en inspanning zou besparen. Van belang is, of het verloop van de oestradiolspiegels zou kunnen worden gebruikt als diagnostische test op ovariële veroudering. Tegenwoordig wordt een clomifeenbelastingstest gebruikt, waarbij, na toedienen van 100 mg clomifeen citraat per dag gedurende cyclusdag 5-9 een FSH bloedspiegel op cyclusdag 10 die een bepaalde grenswaarde overschrijdt, wordt gebruikt als een kenmerk voor de kwaliteit van de functie van het ovarium. De waarde van deze test is echter omstreden (Bancsi *et al.*, 2001). Mogelijk kan het toevoegen van de oestradiolrespons op cyclusdag 7 of 8 de voorspellende waarde van deze test verhogen. Licciardi *et al.* (1995)

vonden dat relatief hoge oestradiolwaarden op cyclusdag 3 binnen dezelfde cyclus een lage eicelopbrengst en lage zwangerschapscijfers na een IVF behandeling konden voorspellen. Evers *et al.* (1998) toonden aan dat normale vroeg-folliculaire FSH spiegels in combinatie met hoge oestrogeenspiegels, in een voorafgaande cyclus, een lage respons op stimulatie voor IVF voorspelden. In een alternatieve clomifeenbelastingstest zoals hierboven voorgesteld, zou het van voordeel kunnen zijn, de oestradiolwaarden op cyclusdag 3 (voorafgaand aan clomifeen citraat) te combineren met die op cyclusdag 7-8. De vierde doelstelling van het proefschrift was het verkrijgen van referentiewaarden voor het al of niet normaal zijn van een cyclus, vanuit een groep relatief jonge vruchtbare vrouwen (22-34 jaar oud) met een regelmatige menstruele cyclus, en na te gaan of de follikelgroei, ovulatie, endometriumontwikkeling en hormonale patronen van oudere vrouwen (41-46 jaar oud) verschillen van de referentiegroep, en als dat zo blijkt te zijn, of zulke verschillen de leeftijdsafhankelijke daling van de vruchtbaarheid kunnen verklaren, of eraan kunnen bijdragen. De vrouwen van beide groepen waren in het verleden binnen een jaar zwanger geworden. De oudere groep bestond uit vrouwen in een leeftijd waarop kan worden aangenomen dat de vruchtbaarheid in sterke mate is afgenomen. De jongere vrouwen waren op een leeftijd waarbij de vruchtbaarheid nog optimaal is, of slechts licht verminderd. De afmetingen en de groei van de follikels werden in het verloop van de cyclus gevolgd met transvaginaal echoscopisch onderzoek, en de bloedspiegels van verscheidene hormonen werden gemeten. Het bleek, dat duidelijk abnormale cycli weinig voorkwamen: in de oudere groep 2, vergeleken met 1 bij de jongere vrouwen. Dit betekent dat cyclusstoornissen zoals LUF en andere subtiele cyclusstoornissen niet de verklaring vormen voor de leeftijdsafhankelijke afname van de vruchtbaarheid. Er werden enkele verschillen gevonden tussen de leeftijdsgroepen. De totale lengte van de cyclus was kleiner bij de oudere vrouwen, hetgeen werd veroorzaakt door een kortere folliculaire fase. Tot nu toe werd gedacht dat zo'n kortere folliculaire fase het gevolg is van een snellere groei van de follikels, die start in de vroege folliculaire fase ('versnelde' follikelontwikkeling). Onze resultaten geven echter aan, dat er geen verschil is in het tijdsverloop waarin de follikels worden gerekruteerd en gestimuleerd tot verdere groei, de dominante follikel wordt geselecteerd, en de ovulatie plaatsvindt. Het verschil tussen oudere en jongere vrouwen is een

*verschuiving in de tijd* van twee tot drie dagen in de ontwikkeling van de follikels en de oestradiol stijging, die al begint tijdens de laat-luteale fase van de voorafgaande cyclus. Onze resultaten tonen duidelijk aan dat er een 'vervroegde' follikelontwikkeling in plaats van een 'versnelde' ontwikkeling is. De eerder optredende stijging van de FSH spiegels in de oudere groep, die al start tijdens de luteale fase van de voorafgaande cyclus zou heel goed de trigger voor een eerder startende groei en verdere ontwikkeling van de follikelgroei kunnen zijn. Onze hypothese hiervoor is dat de eerdere stijging van het FSH wordt veroorzaakt door een verminderde productie van inhibine A door het corpus luteum van de oudere vrouwen (Danforth *et al.*, 1998, Santoro *et al.*, 1999). Het is bekend dat inhibine selectief de productie van het FSH door de hypofyse kan verlagen door middel van een negatief terugkoppelingsmechanisme. Oestradiol en progesteron hebben beide een negatieve terugkoppeling op het FSH (en LH), maar de oestradiol en progesteronspiegels verschilden niet tussen beide groepen tijdens de luteale fase. De sterke daling van het progesteron aan het eind van de levensduur van het corpus luteum, is verantwoordelijk voor het begin van de menstruatie (Le Nestour *et al.*, 1993). Daardoor kan de eerdere stijging van het FSH de ontwikkeling van de follikel vervroegen zonder dat de menstruatie vervroegd optreed. Een ander verschil tussen de leeftijdsgroepen was een afnemende groeisnelheid van de dominante follikel gedurende de drie dagen die voorafgingen aan de ovulatie bij de oudere vrouwen, hetgeen resulteerde in een kleinere doorsnede voor de ovulatie. De oestradiolspiegels en de dikte van het endometrium verschilden niet in dit stadium van de cyclus. Het wordt algemeen aangenomen dat de afname van de vruchtbaarheid met de leeftijd het gevolg is van een afname van de eicelkwaliteit, door een toeneming van het optreden van chromosomale afwijkingen. Echter, minder gunstige omstandigheden tijdens de ontwikkeling van de follikel zouden hiertoe kunnen bijdragen. De vroegere start, tijdens de voorafgaande luteale fase, zou kunnen plaatsvinden onder suboptimale hormonale omstandigheden. De kleinere doorsnede van de follikel voorafgaand aan de ovulatie zou een weerspiegeling kunnen zijn van zo'n minder gunstige start. Andere oorzaken waardoor de follikelkwaliteit verminderd zou kunnen zijn is het kleinere aantal rekruteerbare follikels (als gevolg van een afgenomen voorraad rustende follikels) tijdens het 'FSH-venster', wat zou kunnen leiden tot een blootstelling van deze follikels

aan FSH die te kort van duur is voor een optimale verdere ontwikkeling (Warburton, 1989). Bovendien kan selectie van een dominante follikel uit een kleiner cohort, uitsluitend door toeval, resulteren in de groei van een minder goede follikel. Het is verleidelijk om te veronderstellen dat de vervroegde follikelontwikkeling bij oudere vrouwen kan worden gemanipuleerd. Le Nestour *et al.* (1993) toonde aan dat de vroeg-folliculaire FSH stijging kan worden uitgesteld door het toedienen van fysiologische hoeveelheden oestrogenen. De voordelen van zo'n procedure zouden kunnen zijn: een verbeterde timing van de postcoïtum test (De Ziegler *et al.*, 1999) of een verbeterde timing van intra-uteriene inseminaties (De Ziegler *et al.*, 1998; 1999) Nog een suggestie is dat bij het begin van een hyperstimulatiecyclus voor IVF het endogene en exogene FSH beter zouden kunnen worden gesynchroniseerd (De Ziegler *et al.*, 1998). Het zou ook mogelijk kunnen zijn de vervroegde FSH stijging bij oudere vrouwen vanuit de laat-luteale fase van de voorafgaande cyclus naar voren te schuiven in de vroeg-folliculaire fase, de normale situatie bij jongere vrouwen. Of de follikelgroei en de hormonale processen dan meer op die van jongere vrouwen zouden lijken, en of dit tot een meer vruchtbare cyclus leidt, zou het onderwerp van verder onderzoek kunnen zijn.

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## CURRICULUM VITAE

Pieter van Zonneveld werd op 14 februari 1948 geboren in de goede stad Leiden. Hij behaalde in 1966 'met lof' het diploma HBS-B aan het Rembrandt-Lyceum. In het laatste schooljaar kwam hij in contact met de arts-viroloog dr. N. Masurel, hoofd van het Laboratorium voor Respiratoire Virologie van het Academisch Ziekenhuis Leiden (later hoogleraar aan de Erasmus Universiteit te Rotterdam). Door diens voorbeeld koos Pieter voor de studie geneeskunde. Hij studeerde aan de Rijksuniversiteit Leiden, waar hij op 29 oktober 1971 zijn doctoraaldiploma en op 18 januari 1974 zijn artsenbul kreeg uitgereikt. In de academische vakanties werkte hij aan diverse projecten mee op het Virologisch Laboratorium te Leiden, later ook te Rotterdam. Met de artsenbul in de hand koos hij, ondanks zijn liefde voor de Medische Microbiologie, voor een loopbaan in de directe patiëntenzorg. Na een jaar als algemeen assistent in het St. Barbaraziekenhuis te Geleen werkte hij, enthousiast geworden voor de Verloskunde en Gynaecologie, nog een jaar in dit ziekenhuis onder leiding van de gynaecologen dr. C.W.J. Belder en J.A. Zandvoort. De specialisatie tot gynaecoloog werd gestart op 1 mei 1976 in het Westeinde Ziekenhuis te 's-Gravenhage. Hij werd opgeleid door dr. J.C. Seelen (A-opleider) en mevr. J.C. Been (chef de clinique). In deze periode trad hij in het huwelijk met Marie-Anne Neecke. Vanaf 1 mei 1980 ontving hij zijn B-opleiding in het Bronovo-Nebo Ziekenhuis te 's-Gravenhage, met als begeleiders dr. N.D. Bessem (B-opleider), P.H. Kolkman en M.R. Mackenzie. In dit jaar werd zoon Floris in het Bronovo Ziekenhuis geboren. Op 1 mei 1981 werd Pieter in het specialistenregister ingeschreven. Gedurende de vier volgende jaren nam hij waar in diverse perifere praktijken Verloskunde en Gynaecologie. Onder andere zette hij voor langere tijd de praktijk voort van W.F. Ho Kang You in het Horacio E. Oduber Hospitaal te Oranjestad, Aruba, Nederlandse Antillen. Om meer ervaring op te doen in de Gynaecologische Oncologie werkte hij ook enige tijd in het Antoni van Leeuwenhoek Ziekenhuis te Amsterdam, onder leiding van E.J. Aartsen en dr. J.V.T.H. Hamerlynck. De periode november 1981 – januari 1982 bekwaamde hij zich in de Gynaecologische Microchirurgie op de afdeling van prof. dr. I.A. Brosens aan het Academisch Ziekenhuis St. Raphaël te Leuven, onder leiding van prof. dr. W. Boeckx, prof. dr. S. Gordts en mevr. Lieve van den Maegdenburgh-Desmet. Tijdens deze stage werd in

het St. Raphaël Ziekenhuis dochter Fleur geboren. Tijdens de verdere periode van waarnemen volgde de geboorte van dochter Mirit, in het Bronovo Ziekenhuis te 's-Gravenhage. Op 1 mei 1985 begon Pieter van Zonneveld als 'fellow' op de afdeling Voortplantingsendocrinologie en Vruchtbaarheidsonderzoek (VEVO) van het Academisch Ziekenhuis Vrije Universiteit te Amsterdam. Hier werd hij verder opgeleid in het subspecialisme Fertiliteit, onder leiding van prof. dr. J. Schoemaker, mevr. M.E. Boer-Meisel (tubachirurg) en dr. P.G.M. Hompes (chef de policlinique). Ook in het VU ziekenhuis, werd dochter Jasmijn geboren. Op 1 mei 1987 maakte Pieter de overstap naar het Universitair Medisch Centrum Utrecht, waar hij sindsdien staflid is op de afdeling Fertiliteit en Voortplantingsendocrinologie (FENDO).