DISTURBED GROWTH AND SELECTION OF THE HUMAN OVARIAN FOLLICLE

GESTOORDE FOLLIKELGROEI EN SELECTIE IN HET HUMANE OVARIUM

PROEFSCHRIFT

TER VERKRIJGING VAN DE GRAAD VAN DOCTOR
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With the passage of time some overtly simplistic hypotheses may be adjuged to be incorrect. However, I believe it is of little consequence whether the ideas we treasure today survive the more rigorous criteria of tomorrow. What I believe is of greater importance, is that once in a while we distance ourselves from our specialised interests and attempt to devise some overall concept of how biological systems may operate.

Kenneth P. McNatty

Scientific theory has as its ultimate goal the understanding and prediction of natural phenomena. At cyclic intervals, in the largest half of this world population, more precisely in the ovaries of most of this largest half, a most fascinating phenomenon does take place. A cohort of quiescent follicles accomplish their fate by being recruited to grow towards a common aim, being The One which will ultimately be selected as a dominant follicle to pursue onwards to ovulation. Both recruitment of a cohort of follicles and selection of a dominant one are still poorly understood processes, neither are the reasons of apparently disturbed selection in a number of infertile women characterised by abnormal menstrual cycles. Kenneth McNatty has performed pioneer work to accept the challenge of a better understanding of ovarian function. More important, he transcended the experience of his work in a statement which is a model of common sense and modesty. In complete agreement with the philosophical concept of research he committed to, studies presented in this thesis were performed to contribute to the appraisal of normal and disturbed ovarian follicle growth in the human.

Thierry D. Pache
Rotterdam, the 11th of June 1992

A mes parents,
Renée, Basile et Quentin

Cover: Flora, JAN MASSYS (c. 1500-1575), painting on wood, Hamburger Kunsthalle

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Contents

Chapter 1

Introduction and definition of study objectives

In the year 1672, in Delft, the Dutch physician Reinier de Graaf (1641-1673) described for the first time the differentiation of ovarian follicles into corpus luteum (Jocelyn and Setchell 1972). Reinier de Graaf also provided evidence that the "female testis" played a central role in female reproduction, precising in " De mulierum organis generationi inservientibus tractatus novus" that this organ was effectively an ovary (named after the word "ovarium" used by Fabricius) (Evers and Heinemann 1990). Three hundred years later, our knowledge of the structural evolution of follicles is still inferred from morphological studies. However, very few authors have performed histological studies to address the issue of ovarian follicle growth in women (Block 1951a; Block 1951b; Block 1952; Gougeon 1981; Gougeon 1983; Gougeon 1987; Chikazawa et al. 1986). This is possibly due to the fact that collecting human ovaries at timed stages of the cycle is problematic. Moreover, a tremendous amount of work and time is necessary to achieve quantitative studies of follicle development based on serially sectioned ovaries. Most convincing results - detailed in the next chapter - were obtained by Gougeon (1981). He proposed that follicle life span may extend up to 85 days between the moment a theca interna appears - i.e. at the secondary follicle stage - and ovulation. The major drawback of morphological studies is the cross-sectional design. Attention should therefore be focused on ways to investigate *in vivo* at least part of the anatomical findings.

Structural changes reflect over and over again repeated mechanisms within the ovary, which take place from infancy to menopause (Peters 1969). Cyclic ovarian activity depends on an endocrine system involving the hypothalamus, the pituitary and the ovary. Feedback mechanisms ensure that hormonal secretion is judiciously regulated in order to allow ovarian follicle growth (Neil et al. 1967; Midgley and Jaffe 1968; Yen et al. 1970a; Knobil 1980). Accordingly, any disturbance along the hypothalamo-pituitary-ovarian axis may potentially damage this delicate harmony, and lead to follicle growth arrest and anovulation (Shearman and Cox 1966; Yen 1980; Seibel 1984a). The mechanisms by which pituitary gonadotropins, follicle-stimulating hormone (FSH) and luteinizing hormone (LH), regulate follicle maturation have been extensively investigated, but the process by which only one single follicle is selected to mature during each cycle has not been elucidated. A first difficulty was to identify which parameters might be used to assess whether a follicle had been selected. With this objective in mind, a number of investigators have paid attention to size and hormonal content of follicles. During the mid to late follicular phase, it was noticed that 17-ß estradiol (E2) levels were higher in venous plasma draining the ovary bearing a follicle $\geq$ 10 mm in diameter, and that this follicle contained a much higher concentration of E2 as compared to any follicle < 10 mm collected at the same time (Baird and Fraser 1975). More precise information was provided by McNatty and colleagues (1979a; 1979b; 1979c). They observed that for any given size a follicle had

a determined maximal number of granulosa cells, and that the concentration of E2 in follicular fluid correlated positively with this granulosa cell number. When the percentage of this optimal cell number was above 50%, the follicle was regarded as being healthy. When the percentage fell below 50%, the follicle could be considered as atretic. The observation that the healthy or atretic state of a follicle could be defined through its intra-follicular steroid content enabled McNatty to describe evolutionary stages of follicular maturation, and to speculate about the period of selection. He observed that in the early follicular phase, follicles as small as 4 mm in size could be considered as healthy, and were thus candidates for further evolution to dominance. The same author (McNatty et al. 1983) later estimated that the diameter at which one follicle could be selected ranged between 4.0 and 6.5 mm, whereas in two other morphological studies in the follicular phase, reported sizes were between 5.5 and 8.2 mm (Gougeon and Lefèvre 1983), and 4.7 mm (Chikazawa et al. 1986). Altogether, it may be inferred that a size of 10 mm or more is likely to represent an upper limit above which any follicle may be considered as having been selected, and consequently called dominant. Under this diameter, size alone is not sufficient to label a follicle as such. The term "dominant" was introduced by Hodgen (1982) to describe the selected follicle which, for the duration of one cycle, first as a follicle, second as a corpus luteum, will dictate the course of events in the hypotalamo-pituitary and ovarian axis.

Before introducing further considerations about integrated mechanisms of selection, Baird and Fraser (1975) and McNatty (1979b; 1983) observations must be viewed in the context of the concept of endocrine mechanisms driving follicle growth. In the "two-cell two gonadotropin" model of ovarian follicle development, LH-induced thecal-interstitial cell androgen synthesis is considered to provide the necessary substrate for FSH-induced granulosa cell aromatase activity. This will lead to ovarian estrogen production and subsequent follicular maturation (Short 1962). It has been suggested that the FSH intercycle rise is the mechanism which triggers only one small antral follicle, sized 2 to 4 mm in diameter, to gain dominance about co-growing follicles of the same wave (Baird 1983). In the present thesis, the expression "wave of follicles" will describe the number of maturing follicles which have become gonadotropin dependent at the same period of time of their evolution during folliculogenesis, and gaining of dependency will be termed "recruitment" of follicles. Follicle maturation is not only regulated by endocrine signals. Mechanisms generated within the ovary itself seem to be involved as well. In recent years, a picture has emerged that besides the classic endocrine control of ovarian function, a more complex regulatory system including paracrine (control by neighbouring cells) and autocrine (control by the same cell) modulatory mechanisms is implicated (Hsueh 1986; Findlay and Risbridger 1987; Adashi et al. 1985; Fauser and Hsueh 1988; Tonetta and DiZerega 1989). This system could be involved in the maturation process of a single follicle in each menstrual cycle. However, many important beliefs in ovarian function - more precisely how follicle growth might be tuned at the ovarian level - have been gained *in vitro* using cell cultures, and in most cases in non-primate animal models (Hsueh et al. 1984; Greenwald and Terranova 1988; Richards and Hedin 1988).

Transposition of this knowledge from animal to human, and application of these *in vitro* data to the *in vivo* physiological state must be performed with extreme caution for several reasons. Firstly, much work has been done in various animal species with profound differences in endocrine and local regulation, and different lengths of follicular phase as compared to the human. Secondly, dynamics of follicle maturation implies progressive theca and granulosa cell differentiation. Consequently, what is observed in cultured cells primed - mainly by gonadotropins and growth factors - *in vivo* for a given period of time, is likely to correspond to reality for a limited period of cellular differentiation only. Clearly, cells collected, either on the first, or on the tenth day of the follicular phase are different, and they will not yield the same observations in culture. This issue is particularly relevant in the context of studies of the aromatase activity which plays a pivotal role in E2 biosynthesis, but has been reported as being virtually absent in human follicles < 8 mm (Erickson et al. 1979). Altogether, even if numerous studies using animal thecal and granulosa cell cultures have described the role of growth factors in several steps of follicle maturation, mechanisms of intra-ovarian regulation of follicle growth and selection remain to be established *in vivo* .

Problems of anovulation are a major factor of female infertility. Polycystic ovary syndrome (PCOS) is the most frequent anovulatory disorder in women, and may be characterized by disrupted follicular growth in abnormally large ovaries, by abnormal levels of circulating sex steroid hormones, elevated LH concentrations, hirsutism, and obesity. None of these traits is always present, the clinical picture is therefore heterogenous. Consequently, a consensus for pathogenesis, diagnosis, and treatment of PCOS is absent (Vaitukaitis 1983; Franks 1989; Editorial [Lancet] 1990). Modern sonographic techniques, allowing non invasive examination of the pelvis, looked very promising. However, although sonography has been widely used for the diagnosis of polycystic ovaries, no agreement has been met for criteria of structural changes (Fauser et al. 1991b; Fox et al. 1991). More recent developments in the field of sonography, chiefly the advent of high-frequency vaginal probes allowing depiction of small follicles, should help to provide a more accurate description of polycystic changes. It may be expected that more precise observations of the ovarian structure will enable more detailed correlations with clinical and endocrine findings.

It was the goal of this thesis to transpose part of the above mentioned basic notions to clinical practice. Three ways to stick to studies of follicle growth *in vivo* have been employed. Firstly, the ability transvaginal sonography has to appraise the dynamics of follicular growth, its potential in gaining insight into ovarian physiological and pathophysiological mechanisms was investigated. Secondly, characterization of the endocrine findings in peripheral blood was performed. Thirdly, follicular fluid could be harvested from human ovaries. Intra-follicular hormonal estimates could be correlated to ovarian structure, clinical informations, and sex steroids and gonadotropin levels in serum.

1.2 Outline of the thesis

The **first objective** of this study was to review some aspects of current knowledge of ovarian function in normal menstrual cycles. A discussion of the anatomy and physiology of the normal ovary can be found in chapter 2.

The **second objective** was to provide new information about the dynamics of follicular growth in the normal menstrual cycle, and to validate the use of high-frequency transvaginal sonography for this type of investigation. This is presented in chapter 3.

The **third objective** was to provide an overview of present concepts related to the polycystic ovary syndrome pathogenesis. To this end, clinical, pathophysiological, and morphological findings associated with polycystic ovaries are dealt with in chapter 4.

Attempts to establish an experimental model of polycystic ovaries have failed in different animal species. The **fourth objective** was to find a human model for the study of PCOS. To this end, a morphological study performed in female to male transsexuals is presented in chapter 5.

The **fifth objective** was twofold. Firstly, to analyse the data obtained by using transvaginal sonography in the appraisal of ovarian structure in women with irregular cycles. Secondly, to use the same validated method in a attempt to discriminate between normal and polycystic ovaries. These issues are discussed in chapter 6.

The **sixth objective** was also twofold. Firstly, to assess gonadotropin disturbances in serum in women with the polycystic ovary syndrome as compared to a group of women with cycle abnormalities, and a group of regularly cycling women. Secondly, the aim was to appraise these clinical and biochemical findings in the light of our sonographic criteria for polycystic ovaries. The **seventh objective** was to determine to what extent intra-ovarian steroid perturbations could be present in women with polycystic ovaries. All data concerning these last two objectives can be found in chapter 7.

Conclusions which may be drawn from studies performed in this work are presented in the **general discussion**, in chapter 8.

Chapter 2

Anatomy and function of the normal ovary

2.1 Introductory remarks

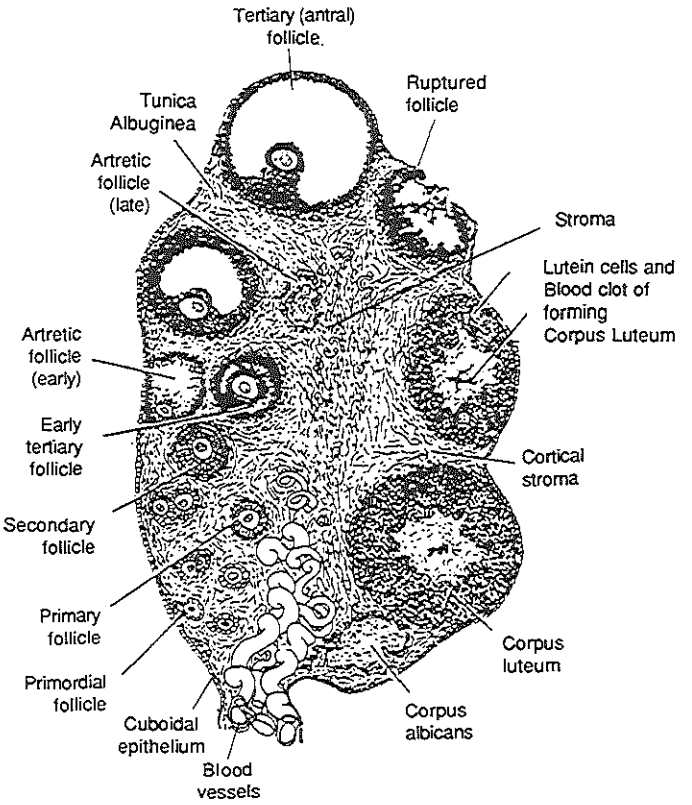
Normal cyclical ovarian function depends on a dynamic feedback system involving the hypothalamus, the pituitary and the ovary. In response to pulsatile stimulation by GnRH, the anterior pituitary will secrete FSH and LH, which will next stimulate ovarian activity. Ovarian gonadotropin-dependent steroid production will in turn modulate GnRH, LH, and FSH secretion. Under physiological circumstances, the cyclic nature of the interplay between gonadotropins and steroids is reflected in the process of follicular maturation, ovulation and formation of the corpus luteum. Formation of follicles may be viewed as a hormone-dependent differentiation and proliferation process (Erickson 1986b). It is the goal of this chapter to review aspects involved in a harmonious development of follicles, with particular emphasis on the roles played by pituitary and ovarian steroid hormones.

2.2 Ovarian morphology

Differentiation of the indifferent gonad into an ovary takes place during the second month of fetal life (Simkins 1928). After the fifth month, the fetal ovary is composed of three different regions, (1) a rete ovarii (hilum) (Simkins 1933), (2) a central medullary portion, and (3) an outer cortical portion (germinal epithelium and stroma). The hilum, at the point of attachment of the ovary to the mesovarium, contains nerves, blood vessels, and steroid hormone secreting hilar cells (Ross and Schreiber 1986). The outermost cellular layer of the cortex is the germinal epithelium. In the adult ovary, this covering epithelium consists of a unicellular layer of cuboidal or squamous cells. Immature germ cells - the oocytes - enclosed in cellular complexes - the follicles - are found in the inner part of the cortex, embedded in the stroma. At least three types of cells have been identified in the stroma: connective tissue and contractile cells, and interstitial cells. Some of these interstitial cells may represent "dedifferentiated" theca cells, remnants of the follicular atresia process. Interstitial cells may secrete androgens in answer to high levels of LH (Mosman et al. 1964), or in response to high levels of hCG during pregnancy (Starup and Visfeldt 1974a). In early stages of ovarian development oocyte nuclei lay close together in cell nests without

distinct cell membranes. The transformation of oocyte cell nests to individual follicles is defined as folliculogenesis, and begins in embryonic life in the human. Follicle transformation always begins in the part of the ovary where the oocytes are in contact with the rete ovarii (Peters and McNatty 1980). In case the rete ovarii is removed before folliculogenesis is started, follicles for some unknown reason will not form (Byskov 1974).

During development of the follicle, the oocyte and surrounding cells differentiate. A morphological classification of these progressive stages of development has been established.



The ovary, modified from New Ham Textbook

A primordial follicle consists of an oocyte in prophase of the first meiotic division, surrounded by a single layer of flattened, spindle-shaped cells. The primordial follicle is separated from the stroma by a basement membrane. In the primary follicle, the spindle-shaped cells become cuboidal. Cells which delineate the granulosa cell

layer begin to differentiate in theca cells (Peters 1979b). These cells, lying in close proximity to the basement membrane, may appear in response to a "theca cell organizer" produced by the granulosa cells (Midgley et al. 1974). In the secondary follicle, these cuboidal cells proliferate to give rise to a stratum granulosum. The stratum consists of two to six layers of granulosa cells, often with several mitoses (Bjersing 1978). After the granulosa has become multilayered, epitheloid transformation of the theca interna becomes apparent. Primary and secondary follicles are sometimes depicted as preantral follicles. Tertiary, also called antral or Graafian follicles are characterized by the progressive formation of intercellular fluid-filled spaces in the granulosa cell layer. These spaces become confluent, giving rise to the antrum. Initiation of antrum formation begins when the follicle is around 150-200 µm in size. Granulosa cell multiplication is the first sign of follicular growth, but above 200 µm in diameter, follicle size will be determined more by fluid accumulation than by cellular proliferation (Bjersing 1978). Atresia is a degenerative process in which all follicular components undergo cytolysis, and may occur at any stage of follicular development. Two million oocytes/primordial follicles are present at birth in the girl. At menarche, not more than 400 to 500.000 are left (Baker 1963). Not more than 400 will be selected to ovulate. This means that most follicles will be lost on their way to development, a loss due to atresia. A number of them may even vanish before growth has started. Once folliculogenesis has been initiated the total number of follicles which degenerate before antrum formation is probably low. In the infant human ovary, only approximately 15% of the growing follicles undergo atresia at a preantral stage (Himelstein-Braw et al. 1976). The total number of follicles in one ovary decreases with age, but ovaries nevertheless increase in size during childhood. Peters (1979) proposed that progressive increase in size of the follicles, in combination with the augmentation of stromal tissue consecutive to follicular atresia were responsible for this increase in ovarian size. After menarche, human ovaries are oval bodies with dimensions of roughly 4 x 3 x 1 cm (Ross and Schreiber 1986). According to the simplified formula for a prolate ellipse - 0.5 x length x width x thickness -(Sample et al. 1977), these values correspond to a volume of 6.0 ml (cm^3). Six ml is most often cited as the upper normal limit for ovarian size in adult women.

2.3 Structural changes during follicle development and selection

The total pool of non-proliferating primordial follicles probably affects the number of follicles that will start to grow. In rodents, a larger number of follicles begin to develop when a larger number of primordial follicles are present (Krohn 1967; Karup et al. 1969). The first step of follicular growth occurs when primordial follicles leave the pool of non-growing follicles, and this is called (initiation of) folliculogenesis. Granulosa cells begin to multiply and the oocyte enlarges (but remains arrested in the prophase of the first meiotic division until just before ovulation). The initiating signal and the mechanisms underlying this event are totally unknown. Folliculogenesis is thought to be a continuous process in the human (Peters 1969;

Peters 1975), and is probably not dependent on gonadotropins (Hisaw 1947; Peters et al. 1973; Erickson 1986). The latter assumption is based on several observations: (1) follicular growth occurs at all ages in childhood (Lintern-Moore et al. 1974), (2) follicles of all sizes up to 6 mm have been found in ovaries from young girls (Peters et al. 1978), and (3) the initial stages of folliculogenesis continue following hypophysectomy (Edwards et al. 1977). Whether a certain level of gonadotropic support is necessary for continued development of follicles once they have started to grow until the antral stage (150-200 µm) has been reached is controversial. On the one hand, small antral follicles have been found in women under oral contraception, i.e. after gonadotropin secretion has been incompletely suppressed for some time (Starup and Visfeldt 1974). On the other hand, Goldenberg and co-workers (1976) reported on five women with the Kallman syndrome (hypogonadism and anosmia) who presented with severely impaired preantral follicular development: three of these women presented with only primordial follicles in their ovaries, one woman presented with one primary follicle and a few cystic atretic follicles, and the last one only presented with a single secondary follicle. FSH may potentially exert effects at a very early stage of follicular growth, since in studies designed to localize binding sites for FSH in the immature rat ovary, it was shown that ^{125}I-FSH binds to follicles at various stages of maturation including the ones with only one or two layers of granulosa cells (Richards and Midgley 1976). Moreover, the same authors also observed that LH receptors were present in the theca interna of the secondary follicles of rodents (Richards and Midgley 1976). Collectively, there is more evidence that cyclical hormonal changes are not necessary to initiate folliculogenesis in the human. However, a modulatory or permissive role of low levels of gonadotropins can not be excluded in preantral stages.

It is not known how much time elapses between primordial and pre-ovulatory stage of the human follicle. In man, the first attempt to quantify follicular growth on a time scale (Block 1951) yielded limited information. Thirty years elapsed before new convincing quantification and qualification of follicular growth was made available (Gougeon 1981a; 1981b; Gougeon and Lefèvre 1983). Analysing the number of preantral follicles, he noticed that this number was fairly constant throughout the cycle. He therefore assumed that mobilisation in the pool of preantral follicles was continuous. As already mentioned by Block (1951), he stated that a normal ovary observed at any moment of the cycle could reveal several follicles between 0.1 and 10 mm in diameter (Testart and Gougeon 1987). The assumption that folliculogenesis is a continuous process, together with the finding of follicles of different size at any stage during the cycle, led Gougeon to postulate the existence of successive waves of growing follicles in the menstrual cycle. In morphological studies, the only possibility to assess follicular growth is to relate a well-defined follicle population in whole ovaries to a precise stage in the cycle. Using this method, Gougeon attempted to reconstruct the waves of growth, to determine the follicle life span from the primordial stage until ovulation. It was then estimated that human preantral follicles need around 85 days to develop to ovulation. The first sixty days (2 cycles) follicle size increases from 150 µm to 1 mm. During the next 2 weeks, equivalent to the luteal phase

preceeding the ovulatory cycle, the follicle destined to ovulate increases in size up to 4-6 mm. The last two weeks, corresponding to the follicular phase of the ovulatory cycle, the follicle which will have been selected will further grow up to an ovulatory size around 20 mm.

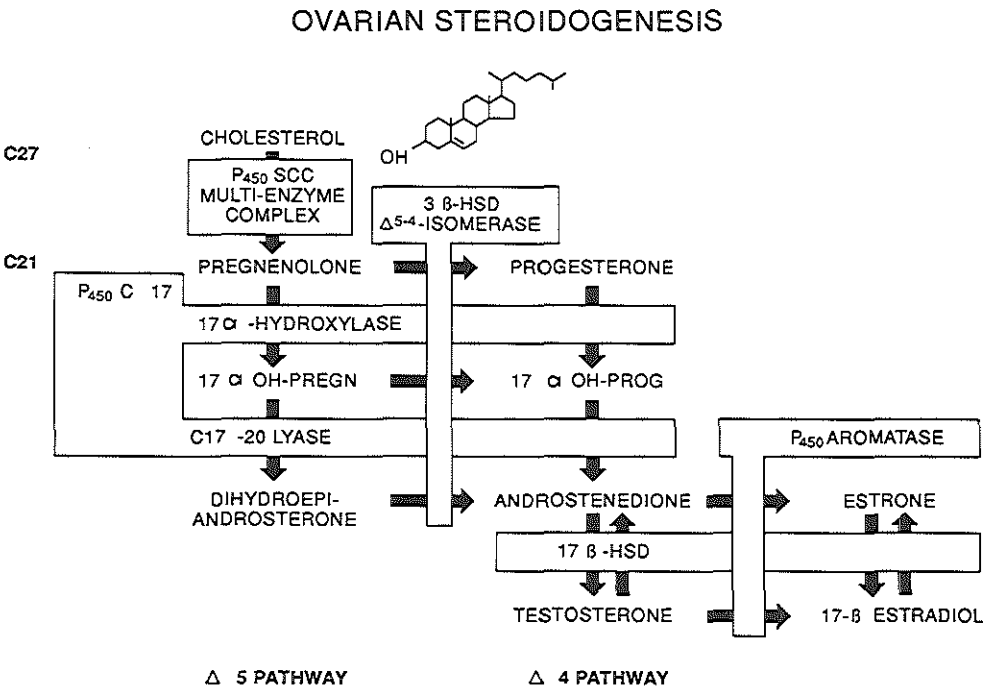
In the late luteal phase, healthy follicles between 2 and 5 mm in size can be observed. Interestingly, the quality of follicles above 2 mm in diameter is highly correlated with circulating levels of FSH (Hillier 1985), and it is generally admitted that follicles larger than 2 mm gain gonadotropin dependency by the end of luteal phase (Gougeon 1991) or early in the follicular phase (Baird 1991). This gaining of gonadotropin dependency is referred to as recruitment. This means that further maturation of the cohort of growing follicles, recruited among the follicles that experience folliculogenesis, will depend on regulation by FSH. However, it has also been noticed that the largest healthy follicle (as determined by a granulosa cell mitotic index) in the late luteal period, could not automatically be viewed as the one that will subsequently be selected, because smaller follicles could have a greater mitotic index. This is in keeping with work performed by McNatty and colleagues (1975), in which they established that very large (some 10 mm and more) follicles occasionally observed in late luteal phase did not appear to sustain their development into the following follicular phase.

Gougeon and Lefèvre (1983) observed that in early follicular phase, the largest follicle presented with such a high granulosa cell mitotic index that smaller follicles were not capable of making up for their growth delay. This could mean that this largest follicle had already been selected to be the one destined to ovulate. The follicle size at which such a finding was made ranged between 5.5 and 8.2 mm, and the period of observation ranged between cycle day 1 and 5. Gougeon's model for follicle growth in the human is convincing indeed, and may fit endocrinological changes in peripheral blood and within follicles. But one has to keep in mind that the limitation of a morphological study is its cross-sectional design.

2.4 Ovarian steroid biosynthesis

An attempt to present an exhaustive review on steroid biosynthesis is beyond the scope of this thesis. For extensive review, the reader is directed to Gore-Langton and Armstrong (1988). Furthermore, pathways of steroidogenesis have been critically discussed recently (Lieberman and Prasad 1991). The aim of this section is limited to summarize steroid biosynthetic and enzymatic pathways, in order to provide a background for presentation of the model of theca-granulosa cell cooperation.

Three classes of steroids are produced by the ovarian follicle, (1) C-21 progestins, (2) C-19 androgens and (3) C-18 estrogens. Their common precursor is cholesterol. Pregnenolone is the precursor for all the steroid hormones, and progesterone is its most abundant C-21 product in the follicle. Progesterone is synthesized as an intermediate product at all stages of follicle development. Testosterone (T) and androstenedione (AD), first identified in follicular fluid of the



mare (Short 1960), are synthesized within the follicle (Ryan and Petro 1966). These two C-19 steroids are immediate precursors of 17-ß estradiol (E2) and estrone (E1), respectively. 5-α dihydrotestosterone (DHT), a 5-α reduced androgen product was also identified in the follicle, but unlike T and AD, DHT can not be aromatized. E2 is ten times more potent than E1 in most biological assays, and is - on a molar basis - the most active of all steroids synthesized in the ovary (Gore-Langton and Armstrong 1988).

The common steroid precursor, cholesterol, is obtained either from blood, or from storage within the ovarian cell, or from local intra-cellular synthesis. The source of cholesterol varies according to the ovarian physiological state (Strauss et al. 1981). Follicles at different stages of maturation have different blood supply, thus the access to circulating cholesterol carriers such as the low density lipoproteins (LDL), is not identical. Theca interna, richly vascularised, has access to LDL products. The granulosa cell layer is isolated from the blood by its basement membrane.

The first step of cholesterol conversion to steroids is rate limiting, and takes place in mitochondria. Cleavage of C20,22 bonds by a multi-enzyme complex including the cytochrome P-450 side-chain cleavage oxygenase ($P\text{-}450_{SCC}$) results in C-21 pregnenolone production. Pregnenolone is next converted to progesterone in the microsomes by an enzyme complex, the "delta^5-3ß hydroxysteroid dehydrogenase (3ß-

HSD): delta$^{5-4}$ isomerase". This reaction is irreversible. A similar enzymatic system converts 17α OH-pregnenolone to 17α OH-progesterone, and dehydro-epiandrosterone (DHEA) to androstenedione (AD).

The rate-limiting step of follicular androgen synthesis involves one cytochrome P-450 enzyme (P-450$_{C17}$). The reaction can use either pregnenolone, or progesterone as substrates. If pregnenolone is used, DHEA is produced following a sequence referred to as delta^5 pathway. When progesterone is the substrate, AD is formed (delta^4 pathway).

An enzyme complex referred to as "aromatase" will convert C-19 androgens to E1 and E2. This complex is a P-450 oxydase system that catalyses three hydroxylation reactions (P-450$_{AROM}$). AD can be converted to T, and T to AD, by a 17ß hydroxysteroid-dehydrogenase (17-ß HSD). The same applies to conversion between E1 and E2. In the human, the main route for ovarian E2 biosynthesis would be through the delta^5 pathway (Ryan and Smith 1965; Lipsett 1978). In keeping with this, formation of AD was reported to proceed mainly via the delta^5 pathway in normal and in polycystic ovaries (Aakvaag 1969).

2.5 Interplay between gonadotropins and steroids during follicle growth: the "two cell-two gonadotropin concept"

Early investigations in swines (Greep et al. 1942) demonstrated that LH and FSH were acting together to stimulate estrogen production. This finding was confirmed in rats, in which it was shown that both cell types of the follicle - theca interna and granulosa cells - and two pituitary gonadotropins, LH and FSH, are needed for estrogen synthesis (Fevold 1941; Falck 1959). This observation led to the "two cell-two gonadotropin" hypothesis of ovarian steroidogenesis, which was originally formulated by Falck (1959). It was conjectured that theca cells, under LH stimulation produced androgens which were subsequently aromatized to estrogens by granulosa cells, under FSH control. The same author demonstrated that theca interna and secondary interstitial cells were the androgen producing cells in the rat ovary (Falck et al. 1962). Later in the sixties, Ryan and Petro (1966) extended the "two-cell two gonadotropin" concept to the human. The same was demonstrated in the porcine ovary (Bjersing 1967). Belief in this concept was reinforced much later by the pioneering work performed by Dorrington and colleagues (1975), who showed that rat granulosa cells in tissue culture aromatized exogenous androgens only when FSH was added to the culture medium. The hypothesis was further promoted by McNatty et al. (1976), who confirmed that granulosa cells in culture can not synthesize E2 de novo, whereas only in those follicles containing detectable levels of FSH in fluid a statistically significant correlation could be established between the number of granulosa cells and E2 levels (McNatty et al. 1978).

In the eighties, in vitro evidence for the "two cell concept" interactions was overwhelming (Dorrington & Armstrong 1979; McNatty et al. 1979; Hillier 1981). However, Channing and co-workers still favored the theca compartment as the

principal source of ovarian estrogens (Channing et al. 1980). Eventually, it was shown (Hillier et al. 1981) that - although both theca and granulosa cells were able to synthesize E2 in vitro - the aromatase activity of the granulosa cell layer was 600-fold higher than that of the theca cell layer. In addition, it was demonstrated in the non-human primate that granulosa cells are the major site of estrogen production, since removing these cells in situ led to eradication of estrogen secretion only in the ovarian vein homolateral to the intervention (Marut et al. 1983). In rodents it was further demonstrated that progesterone produced in granulosa cells was used by theca interna cells to produce androgens. These androgens were then converted to estrogens by aromatase enzymes in granulosa cells (Liu and Hsueh 1986).

The "two cell theory" is still debated in man, and work was recently performed to uncover part of the uncertainties at molecular level. Voutilainen and collaborators (1986) investigated the regulation of P-450_{SCC} (the single regulatory enzyme of cholesterol conversion to pregnenolone) and P-450_{C17} (the only enzyme catalysing pregnenolone conversion to androgens) synthesis by incubating human granulosa cells with gonadotropins. A gonadotropin-specific stimulation of P-450_{SCC} mRNA accumulation in granulosa cells was found, whereas only tiny amounts of P-450_{C17} mRNA were present in the same cells. Using immuno-histochemistry methods in normal human ovarian tissue, Sasano and co-workers (1989) brought direct demonstration that immunoreactivity of P-450_{AROM} (the regulatory enzyme catalysing conversion of androgens to oestrogens) was confined to granulosa cells, whereas P-450_{SCC} activity and P-450_{C17} activity was seen in theca interna cells exclusively. These attempts to localize steroidogenic enzymes in the different cellular compartments of the follicle support the "two cell-two gonadotropin concept" of sex-steroid hormone biosynthesis in the woman, but none of these studies is conclusive. However, growth of follicles up to a pre-ovulatory size without changes in serum E2 could recently be obtained in a woman with isolated gonadotropin deficiency by using recombinant human FSH (Schoot et al. 1992b). Intra-follicular levels of AD and E2 were low as compared to controls. This may convincingly indicate that LH is needed for adequate AD biosynthesis.

Chapter 3

Ovarian follicular growth under normal conditions. Sonography examination findings.

3.1 Introductory remarks

Before determining whether follicular growth and selection is disturbed under any pathological condition, knowledge of how follicular development occurs under physiological circumstances is mandatory. The technique chosen to assess follicular evolution in vivo was ultrasonography, but in former reports no reference was available of the dynamics of normal follicular growth from the very beginning of the menstrual cycle. Therefore, before undertaking investigations of ovarian function in patients who present with cycle disturbances, a study of follicular growth under normal conditions was performed. The aim was three-fold. As a prerequisite before interpretation of the observations - the intention was to picture very small follicles - accuracy and reproducibility of the measurements obtained by 5 MHz transvaginal sonography had first to be validated. Secondly, starting on cycle day one, increase in number and size of ovarian follicles was studied throughout the whole menstrual cycle on a sequential basis. Thirdly, an attempt was made to better define in regularly cycling women the period of time over which selection of a dominant follicle may take place.

As an introduction, previous work related to the appraisal of follicular growth by using sonography has been reviewed.

3.2 Previous sonography reports on ovarian follicular development in regular cycles

The first report on follicular growth in normal menstrual cycles appeared some 12 years ago (Hackelöer et al. 1979). Using static ultrasound equipment with a 3.5 MHz abdominal transducer, only cystic structures above 10 mm in size were regarded as follicles. As a consequence, follicle number could not be studied. A close correlation (r=0.96) between LH peak, follicle size and serum E2 was established in the five days preceding the LH peak. Renaud and colleagues (1980) used a 2 MHz abdominal transducer to investigate normal cycles starting one day 8. Follicles were first visualized from a size of 8 mm, and again only growth of the largest follicle was considered. In keeping with Hackelöer et al. (1979), they noticed that growth of the largest follicle was linear, progressing 2 to 3 mm a day until ovulation. Interestingly,

in these first two publications, concern about correct identification of what should be considered as a follicle was expressed, illustrating at best technical limitations that were present at that time. Kerin et al. (1981), using static scanning technique, first counted follicles over a five days period prior to ovulation. They identified one dominant follicle and observed 3 or 4 additional follicles between 5 and 14 mm in each ovary. Consequently, 14 mm was elected as a cut-off for dominant or non-dominant follicle size. No difference in growth of non-dominant follicles was observed between the ovary bearing the dominant follicle and the contra-lateral ovary. Surprisingly, attempts to determine accuracy of follicle measurements was not achieved before Wetzels published his thesis on the topic (1983). Addressing the issue in follicles > 10 mm, and using 2.5 or 3.5 MHz transducers, he claimed that accuracy in vitro was higher with static than with real-time scanning methods. Around the same period, accuracy of large follicle sizes estimated by 2.5 MHz transabdominal ultrasound transducer was demonstrated in vivo in women undergoing infertility treatment (Leerentveld et al. 1984). Contrasting with Wetzels observations (1983), a slight supremacy of real-time sector (r=0.75) versus static scanning (r=0.65) in estimating size of follicles above 10 mm was reported.

In an attempt to establish criteria of normality, 12 regularly cycling women were scanned using a 3 MHz transabdominal transducer, starting on cycle day 7 (Eissa et al. 1986). It is a pity that number and size of non-dominant follicles were not taken into consideration. Meldrum and colleagues were the first to report on transvaginal ultrasound scanning of ovarian follicles (1984a), and it became rapidly evident that a giant step towards improved resolution had been made. More recently, transvaginal sonography was demonstrated to be more accurate than the transabdominal route in counting follicles above 8 mm in diameter (Deutinger et al. 1987). That similar conclusions may apply to clomiphene-stimulated cycles was shown by O'Shea and co-workers (1988), whereas no differences in overall or dominant follicular diameters with either technique could be demonstrated by these investigators.

The ovary as a whole may also be considered in ultrasound examinations. Its volume is often calculated using the simplified formula for a prolate ellipse: ½ x length x width x thickness (Sample et al. 1977). Historically, Simkins ovary specimen data (1932) were recalculated by Sample and co-workers (1977), and reported volumes in 4 women between 25 and 30 years old were approximately 6.0 ml. This norm of 6.0 ml is most often used as upper limit for normal adult pre-menopausal ovary (Deutsch and Gosink 1982). Munn and colleagues (1986) used a 3.5 MHz transducer to measure the volume of 28 ovaries from 15 women (mean age 28.5 years, range 18 to 47). A mean (±SD) ovary volume of 6.5 ± 2.9 ml (range 2.1 to 13.8) was reported. The 95% confidence limit was 5.4 and 7.6 ml, and the 99% confidence limit was 5.0 and 8.0 ml. Given the above, it is clear that reference information on ovarian follicular development in regular cycles is by far not complete. Furthermore, efforts should be pursued to validate the very promising transvaginal route.

3.3 Growth patterns of non-dominant ovarian follicles during the normal menstrual cycle

Introduction

Much progress has been made in our knowledge of human ovarian physiology, but the process of selection and dominance of a pre-ovulatory follicle remains poorly understood (Zelznik and Hillier 1984; Tonetta and diZerega 1989). The most interesting body of data has emanated from morphological cross-sectional studies leaving the dynamic dimension to remain speculative (Block 1951; Gougeon and Lefèvre 1983; McNatty et al. 1983; Chikazawa et al. 1986). Using transabdominal sonography, a number of observers have tried to investigate human follicular development in normally cycling women (for review, see Lenz 1985). However, the imaging resolution of this approach was limited in that it did not allow reliable assessment of early changes in size of small follicles. A new perspective is offered by the advent of transvaginal sonography allowing visualization of ovarian follicles as small as 2 mm in diameter.

The objective of the present study was to provide more insight into the process of selection of the dominant follicle as well as growth patterns of nondominant follicles throughout the normal menstrual cycle.

Materials and methods

Subjects and study protocol

Seven normal regularly cycling women participated in this study. Mean cycle length was 27.8 days (range 25 to 31 days), and mean age was 28.0 years (range 24 to 33 years). All subjects had neither received hormonal treatment for at least 12 months prior to the study, nor undergone any type of abdominal surgery. Mean body mass index was 21.7 kg/m^2 (range 19 to 25 kg/m^2). This study was approved by the Ethics Review Committee of the Erasmus University, and written informed consent was obtained from each volunteer.

Serial transvaginal ultrasound examinations were performed on cycle day: 1 or 2, 3 or 4, 5 or 6, 7 or 8, 9 or 10, 11 or 12, 13 or 14, 15 or 16, 17 or 18, 21 or 22, and 25 or 26 (and if necessary 29 or 30). All examinations were performed by the same observer (T.D.P.) at different times during the day, using a 5.0 MHz transvaginal transducer (Orion, Philips Medical System, Eindhoven, The Netherlands). The ovaries were localized in relation to the iliac vessels. Follicles appeared as echo-free, round or ovoid translucent structures. Follicle number was established by scanning each ovary from the inner to the outer margin in longitudinal cross-sections. Follicle size was determined from 2 dimensions (longitudinal and anteroposterior) or from 3 dimensions (longitudinal, anteroposterior and transverse) depending on the longitudinal diameter of the follicle ($\leq$ 6.0, or $>$ 6.0 mm respectively). In each instance the mean of the measurements was taken to be the follicle diameter.

Analysis of the number of follicles and follicle growth patterns was carried out in 3 successive periods of the menstrual cycle: the early follicular phase (from menses up to and including the day of selection of the dominant follicle), the late follicular phase (between the day of selection and the day of the luteinizing-hormone [LH] peak), and the luteal phase (following the LH peak until menses). When a leading follicle could be clearly observed by ultrasound it was assumed selection had taken place since the previous examination two days earlier. The ovary bearing the leading follicle was called dominant ovary.

Accuracy and reproducibility studies

The accuracy of follicle size measurements was established using a multipurpose tissue/cyst phantom (Model 84-317. RMI Middleton WI). Cyst-like structures of 2.0, 4.0, 6.0 and 12.0 mm were measured by ultrasound twice daily for 5 consecutive days. Reproducibility of follicle number and size was tested in all subjects through duplicate measurements at 30 minute intervals on cycle day 6 or 7, 8 or 9 and 10 or 11.

Hormone estimations

Daily blood samples were obtained through venepuncture and centrifuged within half an hour after withdrawal. Serum was stored at -20°C until assayed. Progesterone (P) and LH levels were measured as described previously (de Jong FH et al. 1974; Fauser et al. 1990). P was estimated by radioimmunoassay, using an antibody raised against an 11-α-hydroxyprogesterone-hemisuccinate bovine serum albumin complex. Radio- immunometric assays employing a number of monoclonal antibodies were used to measure LH (Medgenix, Fleurus, Belgium). Intra-assay and inter-assay coefficients of variation were < 16.4 % and < 17.1 % for P, < 3.1 % and < 5.2 % for LH, respectively.

Statistical analysis

The time scale for ultrasound examinations was related to the day of LH peak which was defined as day 0. For clinical purposes, results were expressed according to the first day of the last menstrual period (=cycle day 1) as well.

Least-squares regression was used to estimate growth rates of dominant follicles in individual women. This method was also used to evaluate growth of nondominant follicles during various phases of the cycle. The study of follicle size was essentially longitudinal, but data could not be analyzed as such because individual nondominant follicles could not be identified in subsequent measurements. Friedman's test and the signed-rank test (Wilcoxon) were used for comparison of both follicle growth slopes and number of follicles between different phases of the menstrual cycle. Reproducibility of various parameters in vivo was determined using analysis of variance. P=0.05 (two-sided) was considered the limit of statistical significance. Data are expressed as mean ± standard deviation (SD), unless otherwise indicated.

Results

Hormone profiles confirmed the presence of normal ovulatory cycles with a mean midluteal plasma P concentration of 44.2 ± 15.8 nmol/L. Mean plasma LH surge

concentration was 36.9 ± 10.6 IU/L, and LH peaks were situated between cycle day 13 and 18 (mean cycle day 15.7).

Accuracy and reproducibility studies

The accuracy study showed an underestimation of follicle size with mean values of 0.45, 0.50, 0.20 and 0.15 mm for follicles sized 2.0, 4.0, 6.0 and 12.0 mm respectively. In the in vivo reproducibility study, the number of follicles counted in both sessions coincided in 26 out of 42 observations (3 examinations x 7 patients x 2 ovaries). Both counts differed by one follicle on 15 occasions, and a difference of 2 follicles was noted in one instance. In the duplicate measurements, 10 times a

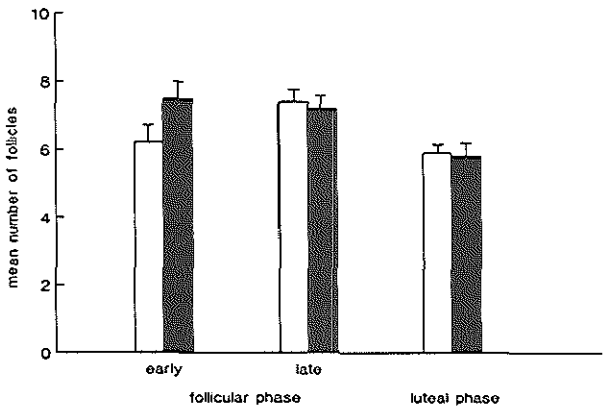


Figure 1 Mean number (±SEM) of follicles in the dominant ovary (□) and in the non-dominant ovary (▩) in seven normally cycling women during the early and late follicular and luteal phase of the menstrual cycle.

dominant follicle was involved. The coefficient of variation associated with the measurement error of these leading follicles was 3%. Individual nondominant follicles could not be labelled, therefore measurements of the size of individual follicles could not be compared. The distribution of follicle sizes obtained at both measurements, however, showed a close resemblance. The coefficients of variation associated with the mean and the SD values of both distributions were 7% and 16% respectively.

Number and size of follicles

The total number of ultrasound examinations per subject varied between 10 and 12. A considerable variation in number of follicles per ovary was observed between subjects for both the dominant ovary (range 3 to 11 follicles) and the nondominant ovary (range 3 to 11 follicles). The mean number of follicles in the different phases of the cycle varied between 5.9 and 7.4 for the dominant ovary, and between 5.8 and 7.5 for the non-dominant ovary (Fig. 1). Differences were not statistically significant. The presence of a dominant follicle could be ascertained between 9 and 6 days before the LH surge (mean 7.4 days), i.e. between cycle days 5 and 12 (mean cycle day 8.3)

at a follicle diameter between 6.5 and 14.0 mm (mean 9.9 mm). At the same time, differences in size between the dominant and the second largest follicle varied between 2.0 and 6.0 mm (mean 4.0). All leading follicles displayed a linear growth rate which ranged between 1.4 to 2.2 mm per day (mean 1.7 mm) until the LH peak was reached. At the time of the LH surge, the leading follicle measured between 18.1 and 22.6 mm (mean 20.6 mm). The diameter of nondominant follicles always remained below 11.0 mm. In the dominant ovary, an increase in mean follicle diameter in the early follicular phase and a decrease in the late follicular and luteal phase could be observed (Fig. 2).

The mean slope for follicle growth was significantly different from zero ($P<0.05$) in all three phases of the cycle for the dominant ovary and only in the early follicular phase for the non-dominant ovary (Fig. 3). In the dominant ovary, there was a significant difference ($P<0.05$) in mean slopes for follicle growth both between the early follicular phase and late follicular phase, and between the early follicular phase and luteal phase.

Discussion

The present study demonstrates that transvaginal sonography permits accurate and reproducible determinations of follicle number and size throughout the menstrual cycle. It should be emphasized, however, that our accuracy study was performed under in vitro circumstances and reproducibility (except for the dominant follicle) could only be tested in a cohort of follicles. Daily estimates of P and LH confirmed the presence of normal menstrual cycles. The LH peak was established between cycle day 13 and 18, reflecting considerable variation in ovulation time among normal menstrual cycles. In a larger study of 68 normally menstruating women, one third displayed a midcycle LH peak before cycle day 12 or after cycle day 18 (Landgren et al. 1980).

The mean number of follicles per ovary showed little variation throughout the menstrual cycle, although a considerable interindividual variation existed. It is of interest that our observation of ovaries containing up to 11 follicles between 2 and 10 mm in diameter in normal menstrual cycles, coincides with criteria of multifollicular ovaries as described in women with weight-loss related amenorrhoea (Adams et al. 1985). Characterization of normal limits for number and size of follicles should also be of value in the diagnosis of polycystic ovaries (Polson et al. 1988).

The leading follicle was first visualized between cycle day 5 and 12 at a mean follicle diameter of 9.9 mm. This is in agreement with a morphology study in which a dominant follicle was first recognized between cycle day 6 and 14 in 8 out of 11 subjects (Chikazawa et al. 1986). In the present study, selection of the dominant follicle was assumed to take place between cycle day 3 and 10. Follicle diameter at the time of selection of the dominant follicle will be below 9.9 mm, and therefore appears not to be essentially different from that reported by others (between 4.0 and 8.2 mm) on the basis of endocrine and histological data (Gougeon and Lefevre 1983; Chikazawa et al. 1986; Mc Natty 1982).

It is noteworthy that in the present study every follicle with a diameter of 11

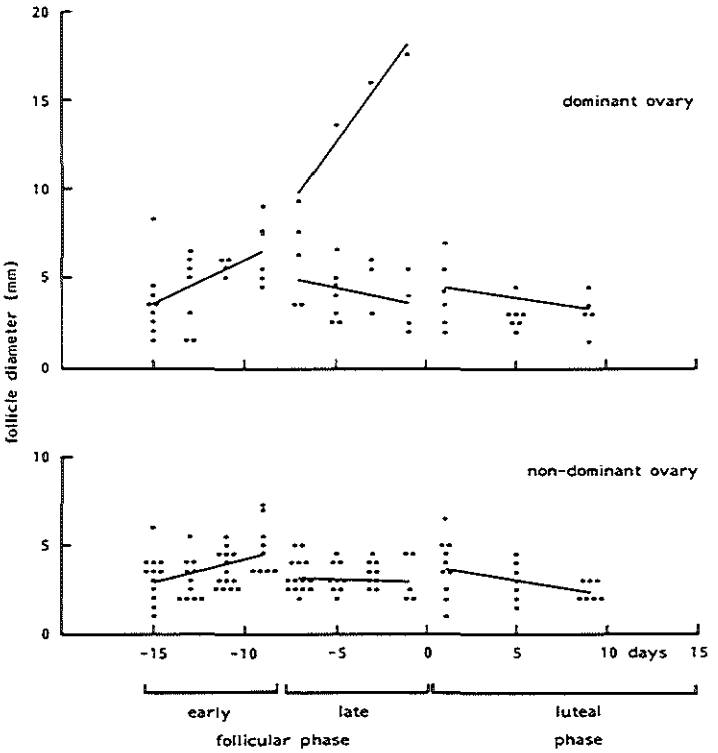


Figure 2 Follicle diameters according to the day of sonographic measurement for both ovaries of one representative normally cycling woman (day 0 = LH surge). Curves denote least-squares regression lines separately for dominant and non-dominant follicles.

mm or more was dominant. Intrafollicular androgen/estrogen ratios are regulated by granulosa cell aromatase activity (Hsueh et al. 1984). It has been shown that aromatase activity in vitro is restricted in follicles up to 10 mm, whereas in larger follicles this activity is greatly enhanced, resulting in high estradiol levels in follicular fluid (Hillier et al. 1980). In addition, a much higher concentration of estradiol was observed in venous plasma draining the ovary containing at least one large follicle > 10 mm in size (Baird et al. 1975). Our in vivo findings seem to substantiate that a follicle diameter of 10 mm acts as a threshold in the further fate of ovarian follicles. Beyond this size, the dominant follicles will continue to grow while nondominant follicles will go into atresia.

During the early follicular phase of the menstrual cycle, both the dominant and nondominant ovary exhibit follicle growth in the majority of follicles. During the late follicular and luteal phase, a decrease in mean size of follicles was established in the

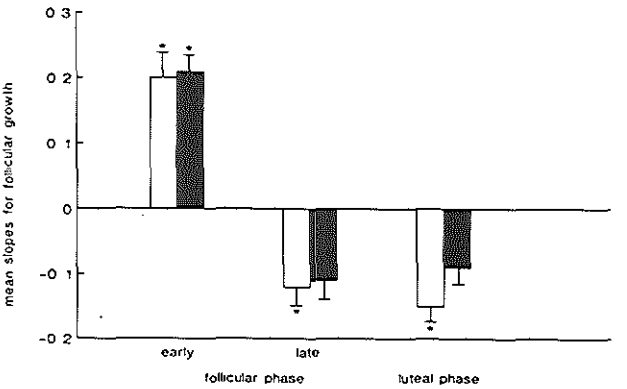


Figure 3 Mean slopes (±SEM) for growth of nondominant follicles in the dominant ovary (□) and in the nondominant ovary (▨) in seven normally cycling women during the early and late follicular and luteal phases of the menstrual cycle. (*) Represents $P < 0.05$ as compared with 0.

dominant ovary only. These observations suggest that suppression of growth of nondominant follicles solely occurs in the presence of a dominant follicle. It may be hypothesized that after selection, a hierarchic relationship exists between the dominant follicle and other follicles of the recruited cohort, indicating that intra-ovarian paracrine mechanisms may be involved, as has also been put forward by others Hillier et al. 1980). These observations also emphasize the limited significance of gonadotropins concentrations in the general circulation after selection of a dominant follicle, since differences in follicle growth could be observed comparing the dominant and the nondominant ovary. This process of selection may be overruled by supraphysiological concentrations of LH and follicle-stimulating hormone (FSH), as seen in induced ovulation using exogenous gonadotropins. Moreover, ultrasound studies have shown that several follicles larger than 10 mm in diameter may be observed in the majority of gonadotropin induced cycles (Seibel et al. 1981). This continuous stimulation of follicle growth may give rise to the ovarian hyperstimulation syndrome and multiple pregnancies.

It can be concluded that transvaginal sonography provides reliable information on follicle number and size. Characterization of limits for normal number and size of follicles will allow more accurate differentiation between normal and abnormal ovarian function. Presented data may also serve as a reference for sonographic monitoring of ovulation induction by exogenous gonadotropins.

Summary

Transvaginal ultrasound examinations were performed in 7 normally cycling women in order to characterize growth of nondominant follicles in both ovaries. Mean

follicle number showed little variation throughout the menstrual cycle with no differences between dominant and nondominant ovaries. Up to 11 follicles (≥ 2 mm) were observed in any one ovary. From observations of the first appearance of the dominant follicle (mean size 9.9 ± 3.0 [standard deviation] mm), selection was assumed to take place on cycle day 6.3 ± 2.3. The diameter of nondominant follicles always remained < 11 mm. Growth of small follicles was established in both dominant and nondominant ovaries up to the time of selection. The late follicular and luteal phase were characterized by a decrease in mean growth slopes of nondominant follicles in the dominant ovary only. These observations may provide in vivo evidence for the concept of intra-ovarian paracrine mechanisms, and may have implications for the sonographic diagnosis of anovulation and monitoring of ovulation induction.

Chapter 4

Morphological, pathophysiological, and clinical aspects associated with the polycystic ovary syndrome

4.1 Introductory remarks

> The elastic picture of the polycystic ovarian syndrome probably reflects more credit on the authors'mental agility than on their experimental vigor.
>
> Joseph W. Goldzieher & James A. Green. 1962

Polycystic ovary syndrome (PCOS) consists of a disorder associated with polycystic ovaries. It is the most frequent ovarian disease in women in their reproductive period. Unfortunately, it remains a poorly defined entity, since several etiological factors may lead to a common end-result: polycystic changes in the ovary. The diagnosis of PCOS itself has become a semantic problem, since in a context of chronic hyperandrogenic anovulation some will choose to label their patients as affected by PCOS without paying attention to the aspect of the ovaries. In no circumstance has evidence been brought that one approach is better than the other. The fact that diagnostic criteria are not identical in all clinical settings has probably weakened therapeutic possibilities. This is of importance, since treatment of infertility associated with PCOS may be difficult, and response to medication is accompanied by high rates of hyperstimulation and early pregnancy losses (Regan et al. 1990).

However general agreement does exist for at least one point: follicular growth is arrested in polycystic ovaries. It is unknown at which evolutional stage of the disease a higher number of small (cystic) follicles can be observed, and what factors

disturb folliculogenesis. These observations indicate that there is a need for a reappraisal of mechanisms and diagnostic criteria of PCOS. Since we are dealing with the polycystic ovary syndrome, and infertility consecutive to anovulation is the major complaint of PCOS women, it has been decided for the present thesis to systematically include the ovarian aspect in criteria used to diagnose the condition. This choice was based on the belief that it should be possible to assess the ovarian aspect in detail, and in all circumstances, by using validated transvaginal sonography methods. To this end, current knowledge about morphology, pathogenesis, and clinical findings which have been associated with polycystic ovaries will be reviewed in this chapter. Sonographic findings will be later discussed in chapter 6.

4.2 Historical aspects

In the year 1450, during the rule of the Emperor Chung Hsi, a chinese author named Ho Tsa Chih may have mentioned for the first time polycystic ovaries in literature (Cooke 1989). In western literature, the first description of a woman with clinical and morphological signs of PCOS was by Antonio Vallisneri in the year 1721 (Vallisneri 1721). He described a "giovane rustica maritata, moderatamente pingue ed infeconda, con due ovaeie più grandi del normale, come un uovo di colomba, bernoccolute, lucenti e biancastre". One century later, Chérau (1844) described gross sclerocystic changes in the human ovary. Waldo (1885) reported upon cystic degeneration of the ovaries, and removal of small cysts to relieve lower abdominal pain in one patient. He estimated that treatment had been unsuccessful, since cystic ovarian changes were observed again at second laparotomy, one year after ovarian partial resection had taken place. Before the turn of the century, some german authors mentioned the existence of bilateral polycystic ovaries, and described variable outcome following cuneiform resection (for review, see Goldzieher and Axelrod 1963). Goldzieher (1981) has mentioned two early extensive discussions about the benefit of wedge resection for cystic degeneration of the ovary, the first one by Finley in 1904, the second one by Goldspohn (1914). The latter author attributed multicystic follicular degeneration to ovarian prolapse caused by uterine displacement and to "evil habits of living", such as incomplete coitus, masturbation, and amenorrhea consecutive to a cold or a mental trauma. Proposed mode of treatment was removal of cysts, ovarian suspension to the broad ligament, and amelioration of life hygiene. Mc Glinn (1916) attributed microcystic ovarian changes to uterine retro-verso-flexion. For treatment, he advised to replace the uterus in a more physiological situation. It was not before the year 1935 that Stein and Leventhal delineated the syndrome named after them. They described the association of bilateral polycystic ovaries and "menstrual irregularity featuring amenorrhea, a history of sterility, masculine type of hirsutism, and less consistently, retarded breast development and obesity" (Stein and Leventhal 1935). During the years following the original description, opinion about the origin of the disease slowly switched from mechanical hypothesis to suspicion of an endocrine disturbance (Stein and Cohen 1939; Geist and Gaines 1942; Stein 1945; Stein et al.

1949; Leventhal and Cohen 1951; McGoogan 1954; Plate 1958; Stein 1958; Leventhal 1958; Stein 1959). In fact, the initial assumption was that follicle cysts overcrowded in ovarian cortex were preventing the normal Graafian follicle from reaching the surface of the ovary. However, already in 1945, Stein suspected that changes were due to a disturbance of hypophyseal function resulting in the formation of numerous cystic follicles. But, others denied this possibility (Ingersoll and Mc Dermott 1950), arguing that FSH values were within normal limits in their cases.

The "two-cell to gonadotropin" hypothesis provided a basis for the understanding of steroidogenesis in the ovary under normal conditions (Falck 1959; Falck 1962). This may have prompted comparative studies of steroidogenic activities of normal versus polycystic ovarian tissue. Accumulation of androgens was demonstrated in human follicle fluid (Short and London 1961), and in human ovarian tissue (Lanthier and Sandor 1960; Axelrod and Goldzieher 1962; Mahesh an Greenblatt 1962), whilst E2 production was almost not detectable. As a consequence, a defective aromatisation of androgens into estrogens was proposed as a likely pathophysiological mechanism in PCOS. Since treatment with FSH led in vivo to a rise of E2 in the ovarian follicle (Short 1964), and to in vitro production of E2 by granulosa cells collected from polycystic ovaries (Erickson et al. 1979), the putative aromatisation disturbance was presumed to be functional. Since then, other models have progressively been put forward to explain the pathogenesis of PCOS. Other hypotheses regarding the etiology of PCOS will be discussed in the following section.

4.3 Pathogenesis

MAIN ETIOLOGICAL FACTORS POSSIBLY INVOLVED IN PCOS

Mechanical factors*
Gonadotropin secretory abnormalities
Ovarian steroidogenic abnormalities
Adrenal androgen excess
Insulin resistance and hyperinsulinemia
Hyperprolactinemia
Genetic factors
Obesity, low SHBG, and peripheral hyperestronemia

* Discussed in chapter 4.4 "Morphology"

4.3.1 Gonadotropin secretory abnormalities

Abnormally high LH secretion is common in PCOS, and may be observed in as much as 70% of the cases, together with low or normal FSH production (Yen et al. 1970b; Rebar et al. 1976). This has been attributed to an exaggerated LH release with

either increased frequency, or augmented amplitude (Rebar et al. 1986, Rebar 1984; Kazer et al. 1987), possibly related to an enhanced pituitary sensitivity to GnRH. Hyperestrogenism is frequent in PCOS patients, and chronically elevated unbound estradiol levels have been reported to augment the pituitary sensitivity to GnRH (Rebar et al. 1976; Baird et al. 1977; Lobo et al. 1981). A primary hypothalamic defect in PCOS has been hypothesized as a result of dissociated dopaminergic and opioidergic inhibitory mechanisms on GnRH neuronal activity (Quigley et al. 1981; Cumming et al. 1984). However, alteration of the hypothalamo-pituitary function in PCOS is probably functional for at least two reasons. Firstly, several treatment regimens have been shown to restore cyclical hypothalamo-pituitary activity in PCOS women (Franks 1989). Secondly, these women have normal responses to negative and positive feedback actions of estradiol (Baird et al. 1977; Chang et al. 1982).

In women, GnRH and sex steroid hormones act together at the pituitary level to regulate the quantity of gonadotropin secretion and their biopotency. McArthur et al. (1958) first described abnormally high urinary excretion of bioactive LH (BIO-LH) in women with the Stein-Leventhal syndrome. Augmented BIO-LH concentrations, and BIO- to immunoreactive LH (I-LH) ratios were further reported (Lobo et al. 1983; Lobo et al. 1984b) in PCOS. Since steroid feedback actions have been reported to affect biopotency and immunoreactivity of gonadotropins in animals (Solana et al. 1980; van Landeghem et al. 1981), it may be hypothesized that the hyperandrogenemic condition in PCOS women together with their hyperestrogenism could induce abnormal regulation of BIO-LH production. Besides, to estimate whether altered BIO-LH levels parallel classically elevated I-LH concentrations may bring additional support to the concept of the importance of LH-induced androgen production within the ovary.

Normal or low immunoreactive FSH levels have been reported by numerous authors. Since abnormally high levels of bioactive inhibin (INH) were found in follicular fluid of polycystic ovaries, this peptide has been suspected to bring about lower FSH levels in PCOS women (Tanabe et al. 1983). This concept could not be confirmed by Buckler et al. (1988), who found similar immunoreactive INH serum levels in PCOS and in regularly cycling control women. However, a normal in vitro response of granulosa cells obtained from PCOS patients (Erickson et al. 1979), and the clinical observation that ovulatory cycles can be induced in most patients using exogenous FSH point to the possibility of disturbed FSH action in this syndrome, probably at the follicular level. In this regard, since no data are available, it should first be determined whether bioactive FSH levels are normal in PCOS.

A LH/FSH ratio above 3 (Lobo et al. 1981), as measured by classical radioimmunoassay (RIA), is widely accepted as one of the diagnosis criteria of PCOS. However, monoclonal antibodies to detect gonadotropins are increasingly used (immunoradiometric assay [IRMA]). Consequently, the validity of this ratio as a diagnostic tool using values obtained from the more recent IRMA method should receive rigorous experimental verification.

4.3.2 Ovarian steroidogenic abnormalities

Much evidence has been accumulated that excess androgen production plays a

key role in the pathogenesis of PCOS. Hyperandrogenemia may originate either from the ovary, or from the adrenal, sometimes from both glands (Lobo 1984; Gonzalez and Speroff 1990). Very often, raised T levels are not suppressed to normal by administration of dexamethasone, which may indicate that the ovary is also a source of augmented androgen production in PCOS. Furthermore, abnormally high AD levels have been repeatedly found in ovarian follicular fluid (Short and London 1961; Giorgi 1963; Jeffcoate et al. 1968; Eden et al. 1990), and in tissue of polycystic ovaries (Lanthier and Sandor 1960; Axelrod and Goldzieher 1962; Mahesh and Greenblatt 1962). Serum androgen concentration could be normalized in women with PCOS, by using GnRH analogs which led to suppression of ovarian function (Chang et al. 1983a; Calogero et al. 1987). Besides, it was recently shown that the ratio of AD to 11ß-hydroxyandrostenedione - a specific adrenal metabolite of AD (Goldzieher et al. 1978) - is elevated in PCOS, further suggesting that the ovary was the major source of excess androgens (Polson et al. 1988b). This excess in ovarian androgens might be due to abnormally high LH stimulation of thecal and stromal compartments (Rebar et al. 1976). Unfortunately, all the above mentioned studies gave no indication as to whether elevated levels of androgens do possibly initiate such polycystic ovarian changes. Furthermore, the direct effects that unusually high androgens may induce at the ovarian level remain to be uncovered. It is known that the number of cystic and atretic follicles is markedly increased in polycystic ovaries (Hughesdon 1982), and androgens have been shown to increase ovarian follicular atresia in rodents (Payne et al. 1958; Louvet et al. 1975). In the human, evidence is lacking that androgens can directly induce polycystic ovarian changes. In a few studies performed in female to male transsexuals who received extended androgen treatment, polycystic-like transformations have been described (Amirikia et al. 1986; Futterweit and Deligdisch 1986; Spinder et al. 1989). But only Amirikia and colleagues (1986) provided some quantified observations.

One may speculate that insufficient conversion of androgens to estrogens may lead to hyperandrogenism. Uncommon primary defects of ovarian biosynthesis of E2 have been reported in association with polycystic ovaries, such as 3 ß-hydroxysteroid dehydrogenase (Axelrod et al. 1965; Barnes et al. 1989), or 17 ß-hydroxysteroid dehydrogenase deficiency (Pang et al. 1987). A dysregulation of thecal cytochrome P-$450_{17\alpha}$ has also been proposed as an etiological factor in PCOS by Barnes et al. (1989b), and Rosenfield et al. (1990).

4.3.3 Adrenal androgen excess

Ovarian and adrenal disease may coexist in PCOS, and the prevalence of this association was initially estimated to be low (Goldzieher and Green 1962). Raj and co-workers (1977) classified patients without evidence of adrenal involvement as "Type I", whereas those women presenting with normal ovarian size and elevated 17 ketosteroids in urine were described as "Type II PCOS". Since an overlap of type I and II was present in a number of patients, they further speculated that unusually high androgen production by the adrenals could lead to ovarian androgenization. Lachelin et al. (1979) demonstrated that dexamethasone suppression followed by adrenocorticotropic hormone (ACTH) infusion were characterized by significantly

greater production of dehydroepiandrosterone, 17-OH pregnenolone, progesterone (PROG), and 17-OH-PROG in PCOS as compared to controls. However, this adrenal hyperresponsiveness could be observed only under maintained adrenocorticotropic hormone (ACTH) stimulation, and it was concluded to the absence of intrinsic adrenal enzymatic abnormality in PCOS. The role that ACTH could play in excess adrenal androgen production was further investigated, and it was shown that plasma ACTH is not different in PCOS as compared to normal controls (Chang et al. 1982b; Horrocks et al. 1983). Congenital adrenal hyperplasia (CAH) due to late-onset incomplete 21α-hydroxylase deficiency (LOHD) (Lobo and Goebelsmann 1980), and an incomplete form of reduced 3-hydroxysteroid dehydrogenase activity (Lobo and Goebelsmann 1981b), were both shown to present with clinical and biochemical features of PCOS. It was confirmed by Chrousos and colleagues (1982) that LOHD could mimic PCOS. They estimated that this disease represents 6 to 12% of hirsute women. However, not every woman with LOHD does display the clinical picture of PCOS (Dewailly et al. 1986). Besides, ovarian theca intersititial cells of CAH patients could be involved in the genesis of polycystic ovarian changes by mechanisms independent of the adrenal glands (Erickson et al. 1989), and ovarian morphological changes may also be independent of the adrenal lesion (Hague et al. 1990). Recently, Fruzzetti et al. (1991) confirmed the adrenal hyperresponsiveness to ACTH stimulation in hyperandrogenic women with high testosterone (T) levels. Interestingly, after administration of a GnRH agonist, which selectively inhibits ovarian androgen production (Chang et al. 1983a), the response to ACTH stimulation test was unchanged. This would suggest that high T levels of ovarian origin do not affect adrenal steroidogenesis (Fruzzetti et al. 1991).

Lobo (1984) reported that high adrenal androgens were associated with elevated free T levels. He hypothesized that E2 might be, in the same way, displaced from SHBG, and result in inappropriate gonadotropin secretion. However, E2 levels have been reported to be within normal limits in most PCOS studies. It was further proposed that a consequence of adrenal androgen excess was excessive hyperestronemia, which in turn led to abnormal gonadotropin secretion and development of PCOS (Loughlin et al. 1986). In an extensive review of the effects of androgens and estrogens on adrenal enzymatic activities, Gonzalez and Speroff (1990) reported that no study had established that circulating steroids were capable of exerting an impact on adrenal steroidogenesis in vivo.

Abnormally high dehydroepiandrosterone-sulfate (DHEAS) levels may be observed in about half of the PCOS patients (Gonzalez and Speroff 1990), but the prevalence of adrenal enzyme deficiencies associated with this syndrome is much lower (New 1988). Thus, that PCOS includes a subgroup of women with adrenal disturbances is established with elevated DHEAS, but underlying mechanisms remain to be uncovered.

4.3.4 Insulin resistance and hyperinsulinemia

In peripheral insulin resistance, exceedingly high levels of insulin are observed together with normal glucose concentrations. In both obese and lean women with PCOS, a strong correlation between fasting insulin and circulating T and AD has been

reported (Burghen et al. 1980; Chang et al. 1983b). The cause-and-effect relationship between hyperandrogenism, hyperinsulinemia, and insulin resistance remains controversial (Barbieri and Hornstein 1988; Dunaif et al. 1990; Poretsky 1991). Obesity alone is also associated with insulin resistance (Peiris et al. 1986), and it was speculated that the association of obesity and PCOS could be caused by insulin resistance or hyperinsulinemia (Barnes and Rosenfield 1989). However, women with PCOS may be insulin resistant irrespective of body mass index (Chang et al. 1983; Dunaif et al. 1987).

High androgens have been implicated as causal factors of insulin resistance because the degree of resistance correlated with androgen concentration (Burghen et al. 1980; Chang et al. 1983; Shoupe et al. 1983). However, hyperandrogenism alone is probably not responsible for high fasting insulin levels, since decreased androgen levels after oophorectomy (Nagamani et al. 1986), or induced by long-acting GnRH analog treatment did not result in significant changes in insulin resistance in PCOS (Geffner et al. 1986; Lanzone et al. 1990).

In vitro studies indicated that insulin stimulates ovarian stromal androgen synthesis (Barbieri et al. 1986). In stroma of normal ovaries, specific binding sites for insulin and insulin-like growth factor I (IGF1) have been demonstrated (Poretsky et al. 1984; Poretsky et al. 1985), whereas in polycystic ovaries there was a decrease in receptors for insulin, with no change in binding sites for IGF1 (Nagamani and Stuart 1990). Since insulin in supraphysiological concentrations can bind to IGF1 receptors (Rechler 1985), it was suggested that under hyperinsulinemic conditions, insulin could exert its steroidogenic action on the ovarian stroma via IGF1 binding sites (Nagamani and Stuart 1990). Barbieri and colleagues speculated that raised insulinemia was pivotal in the development of hyperandrogenism (Barbieri et al. 1988). Their opinion was shared by Barnes and Rosenfield (1989), who viewed insulin resistance as a primary pathogenic factor of PCOS. Recently, support has been brought to their hypothesis by description of type B insulin resistance (secondary to insulin receptor antibodies) associated with hyperandrogenemia in one adolescent female (DeClue et al. 1991). Large doses of insulin were infused, which led to a rise of T and AD, and to an increase of ovarian volume. Insulin resistance spontaneously resolved in this patient, and androgen levels normalized. An additional argument in favor of hyperandrogenemia as a consequence of hyperinsulinemia in PCOS patients was that diazoxide-suppressed serum insulin levels could also result in reduced T levels (Nestler et al. 1989). Also of interest is the fact that hyperinsulinemia may directly decrease peripheral SHBG levels in obese women with PCOS, resulting in an augmented fraction of free T available to target ovarian tissue (Nestler et al. 1991). However, the question remains as how PCOS patients could become hyperinsulinemic. Since endogenous opiate peptides may modulate pancreatic islet function, their involvement in mechanisms leading to hyperinsulinemia and insulin resistance has been proposed (Lanzone et al. 1991). Notwithstanding the absence of agreement concerning the relationship between hyperinsulinemia and hyperandrogenemia, it seems appropriate to say that dysregulation of insulin metabolism is an important parameter in PCOS.

4.3.5 Hyperprolactinemia

The reported prevalence of the association of hyperprolactinemia and polycystic ovaries ranges from 6% (Franks 1989) to 15-20% (Futterweit 1983; Luciano et al. 1984). This variability probably reflects different selection criteria for PCOS patients. Although this association could be fortuitous (Corenblum and Taylor 1982), several mechanisms have been proposed describing polycystic ovaries either as a cause, or as a consequence, of hyperprolactinemia. Firstly, PRL secretion is enhanced by estrogens (Yen et al. 1974), and in PCOS the unopposed action of estrogens on the pituitary together with abnormally high E1 levels could induce hyperprolactinemia. Secondly, use of the dopamine agonist bromocriptin may lower PRL levels and resume regular cycles in some hyperprolactinemic PCOS women (Seibel et al. 1984a; Pehrson et al. 1986). This lends support to the hypothesis that hyperprolactinemia may be a cause of PCOS. Thirdly, hyperprolactinemia may inhibit GnRH activity by affecting dopamine and opioid peptide - neuromodulators of GnRH secretion - concentrations (Quigley et al. 1979; Quigley et al. 1980), and consequently lead to LH dysregulation. However, although PRL pulses are coupled to LH pulses in the normal luteal phase (Braund et al. 1984), bromocriptin treatment in normo-prolactinemic PCOS women did not consistently reduce LH levels or modify LH pulsatility, neither were hyperandrogenemia or anovulation consistently affected (Chapman et al. 1987; Murdoch et al. 1987). Fourthly, high DHEAS levels are common in PCOS, and coincidental hyperprolactinemia and elevated circulating DHEAS levels have been observed, both subsiding under bromocriptine treatment (Carter et al. 1977). This relationship between adrenal steroids and PRL remains to be investigated.

There is also a possibility that the pathogenic role of hyperprolactinemia could be exerted at the ovarian level and directly induce polycystic changes. In the rat, PRL may inhibit LH-mediated androgen secretion (Magoffin et al. 1982), and consequently decrease availability of precursor androgens. PRL may also impede FSH-mediated estrogen production in rodents (Wang et al. 1980; Dorrington and Gore-Langton 1981), which may prevent normal follicular growth. In the human, PRL concentration in follicular fluid of normal ovaries varies with follicle size. As compared to serum levels, PRL concentration is 5 times higher in fluid of small follicles, and similar in the largest follicles (McNatty et al. 1975). PRL inhibits PROG production in cultured human granulosa cells at concentrations similar to those found in fluid of small follicles, but not at concentrations found in fluid of the largest follicles (McNatty and Sawers 1975a). In addition, McNatty (1979a) later noticed that in hyperprolactinemic women (>100 µg/L), PRL levels in follicular fluid were frankly elevated in combination with reduced FSH and E2 levels. Interestingly, mean FSH and E2 serum levels were not affected in these women.

Altogether, these studies provide indirect evidence that PRL could be involved in the regulation of steroidogenesis at the follicular level.

4.3.6 Genetic factors

Chromosomal anomalies, either autosomal dominant (Cooper et al. 1968), or X chromosome linked (De Grouchy et al. 1961; Givens et al. 1971), have been disclosed

in women with clinical signs and symptoms of PCOS. However, the majority has a normal 46XX karyotype (Yen 1980), and patients with X chromosome linked anomalies could represent only a small group. In addition, a genetic deficiency of ovarian 17-ketosteroid reductase - the enzyme converting AD to T - was first reported in three sisters presenting with PCOS (Pang et al. 1987). This was later confirmed as a possible cause of PCOS (Toscano et al. 1990).

Recently, a much higher than generally accepted familial prevalence of polycystic ovaries was reported (Hague et al. 1988). The ovaries of 50 women with PCOS, and of 137 female members of their families were scanned using ultrasonography. In 92% of the available pedigrees polycystic ovaries could be observed, and all daughters of affected mothers were affected. Whether the latter observation could be related to maternal androgens is not known. This large family study strongly suggests that PCOS is a familial disorder, but the use of other markers than ultrasound alone is required to confirm inheritance of the disease.

Givens (1988) group has studied some 20 families, monitoring the distribution of phenotypic findings such as cycle disturbance, hirsutism and hyperandrogenemia. Overall, they demonstrated such a marked variability of all the parameters that pooling of the data was impossible. Accordingly, conclusions drawn from observation of selected families were: 1) there is dominant transmission, 2) the type and degree of expression of the disorder are variable, 3) diabetes mellitus, hyperinsulinemia, obesity, hypertension and ischemic heart disease are present with variable incidence.

Given the above, and in agreement with numerous observations of the literature reviewed by Simpson (1985), it is clearly established that heritable tendencies are detected in PCOS. Nonetheless, the mode of inheritance is unclear. Only identification of primary defects may allow to measure the gene product in all relatives of PCOS patients. In this regard, PCOS symptomatology heterogeneity probably also reflects disparity at the genetic level (Simpson 1991).

4.3.7 Obesity, low SHBG, and hyperestronemia

Obesity was included in the original description of the syndrome (Stein and Leventhal 1935), and was reported to be present prior to puberty in as much as 80% of PCOS (Yen 1980). Furthermore, obesity has often been associated with cycle disturbances and abnormally high androgen levels (Rogers and Mitchell 1952; Axelrod and Goldzieher 1963; Kirschner et al. 1983; Franks 1989). Since weight loss may reverse cycle disturbances in most amenorrheic and hyperandrogenic women (Mitchell and Rogers 1953; Kopelman et al. 1981; Franks 1989), it has been speculated that obesity could play a role in the pathogenesis of PCOS, either through lowering of SHBG (Plymate et al. 1981; Zhang et al. 1984), or via abnormally high E1 levels (Chang et al. 1982; McKenna et al. 1985; McKenna 1988). The decrease in SHBG observed in obesity may result in higher levels of free androgens, and it was suggested that this could in turn lead to inhibition of follicular maturation (Plymate et al. 1981). However, obese women in which SHBG levels were reduced by 30%, and free androgens increased by 70% have also been reported as having regular cycles (Zhang et al 1984).

Chang and co-workers (1982), administered E1 to PCOS and control women, and observed a consecutive decrease in FSH levels, but not in LH levels (1982). The pattern of immunoactive LH and FSH secretion in controls was not altered, whereas FSH but not LH was decreased in PCOS. Since E1 had led to an increased LH/FSH ratio in PCOS, it was inferred that acyclic estrogen production from peripheral aromatization could be responsible for anovulation. Unusually high E1 to AD ratios were further observed in obese amenorrheic women (Loughlin et al. 1985). One additional observation related to the significance of E1 seems of interest (McKenna 1988): follicular growth and ovulation was frequently observed following anti-estrogen clomiphene citrate treatment, in women with normal E2 but elevated E1 levels prior to medication. It was concluded that abnormal gonadotropin secretion was a consequence of hyperestronemia. However, when a test dose of the GnRH agonist nafarelin was administered to PCOS women, E1 rose threefold, while AD increment was only 50% (Barnes et al. 1989b). This unbalanced response of E1, as compared to AD, does not fit with the hypothesis that E1 primarily arises from peripheral AD conversion in PCOS women.

In conclusion, in case indeed E1 plays a role in the pathogenesis of PCOS, it is probably complex. More insight into the incompletely explored regulatory role of E1 on gonadotropin secretion could be envisaged, mainly because estrogens may also affect LH biopotency (Lucky et al. 1979). Tentatively, it could be worthwile to investigate whether bio-active LH is affected by E1 levels in PCOS women.

4.4 Morphology of the polycystic ovary

Stein and Leventhal (1935) originally described bilateral polycystic ovaries as follows: "two to four times the normal size, often maintaining their original shape, but sometimes globular or flattened as an oyster. Cortex was hypertrophied, tunica was thickened, tough, and fibrotic. Cysts were follicular cysts nearly all confined to the cortex, and contained clear fluid. They were from 20 to 100 in each ovary, varying in size from 1 mm to about 15 mm, rarely larger. The color of the ovary was oyster-gray with bluish areas where the cysts were superficial. Corpora lutea were sometimes absent." Other histological considerations involved a sometimes smaller and firmer than normal uterus, and occasional hypertrophy of labia minora and clitoris.

Seven years later, diffuse luteinization of the ovaries was described (Geist and Gaines 1942) in two women presenting with hirsutism, obesity, amenorrhea. In both cases, large nests of vacuolated, luteinized cells were scattered throughout enlarged ovaries, in areas free of follicular structures. These cells were resembling well-developed luteinized theca cells. In addition, the thecal zone around atretic follicles was enlarged, and theca cells showed excessive proliferation - hyperplasia - and luteinisation. Hyperplasia of the ovarian parenchym was also noted. Frankel (1943) elected the term "hyperthecosis" to describe ovaries in which isolated nests of luteinized cells were found in the stroma only. This term was probably recommended because it had formerly been suggested that interstitial cells were arising from the theca

interna layer, and that theca interna cells were becoming luteinized anyhow as follicles were going into atresia (Travis et al. 1965). Nevertheless, groups of luteinized cells in stroma, and hyperplastic and luteinized theca cells around follicles, were progressively incorporated in descriptions of polycystic ovaries (Plate 1958; Vaughan Jones 1962).

A more detailed approach of ovarian morphology was performed by Green and Goldzieher (1965). They used light and electron microscopy to assess changes in wedges of 45 polycystic ovaries. It was found that tunica albuginea germinal epithelium was not different from normal ovaries. However, tunica thickness was more pronounced. This was due to a marked fibrosis, large collagenous fibers, and more unit fibrils of collagen per fiber. In ovarian stroma, fibroplasia associated with an increased amount of density of cells was noted, suggestive of hyperplasia. Nests of luteinized cells were observed in only one case. The population of healthy and atretic follicles, sized 4 to 10 mm, was larger than in normal ovaries. Interestingly, it was not possible to distinguish polycystic from normal ovaries on the basis of thecal histology, i.e. hyperplasia and luteinization were not common features. This is at odds with previous reports (Leventhal and Cohen 1951; Westman 1955; Plate 1958; Jackson and Dockerty 1957; Plate 1960; Vaughan Jones 1962), and was also not confirmed in a large recent series (Hughesdon 1982). These data had the advantage of being obtained from homogeneous material - full-thickness ovarian wedges - as opposed to peripheral (superficial) wedges generally obtained when resection was performed for treatment. In addition, Hughesdon (1982) disposed of 30 age-matched control ovaries which enabled him to carry out quantified comparisons. His most important observations have been listed below.

MORPHOLOGY OF POLYCYSTIC OVARIES
as compared to normal control ovaries

Ovarian volume and surface
Twice the surface of the ovarian cross-sectional area
Thickened ovarian cortex
Marked collagenization of the cortex

Ovarian stroma
Thicker cortical stroma
Marked increase in sub-cortical stroma
Luteinized cell nests in stroma

Follicle number
Unchanged number of primordial follicles
Double number of growing follicles (stage I to III)
Double number of atretic follicles

Pathologists have tried to explain changes found in polycystic ovaries. Mechanical causes - thickened cortex, and ovarian stroma hyperplasia - were first suspected to prevent ovulation. However, polycystic changes may also be observed in women with ovulatory cycles (Plate 1958). In addition, Westman (1955) used silver nitrate to produce thickening of the cortex in rats, and ovulation did still take place. In fact, the hypothesis of the mechanical etiology was born with the history of ovarian wedge resection.

Wedge resection is the surgical procedure by which one segment of ovarian cortex and stroma is removed, and is performed in an attempt to restore normal ovulation. It has been a popular form of treatment until the mid-seventies, and largely abandoned since then because of recurrence of disease, and potential post resection adhesions (Kistner 1969; Buttram and Vaquero 1975; Eddy et al. 1980). Removing ovarian tissue was first thought to suppress a mechanical cause of anovulation. Levels of AD, and T diminished considerably after resection was performed (Mahesh et al. 1978; Katz et al. 1978). Consequently, it was postulated that normalisation of ovarian steroid production following wedge resection caused ovulation.

Already in 1951 Plate had observed ovarian hyperthecosis consecutive to the administration of large doses of human choriogonadotropine (hCG). He therefore postulated that theca interna hyperplasia and luteinisation should be considered as a response to excessive pituitary production of LH (Plate 1958; Plate 1960). Since the number of atretic follicles was found to be abnormally high in polycystic ovaries (Green and Goldzieher 1965), a relationship between the augmented number of atretic follicles and disturbed ovarian steroid biosynthesis was suggested. It was stated that nearly all the morphological features of a polycystic ovary could be explained by an increased LH output (Hughesdon 1982). The same author could not exclude the possibility that LH overproduction would be a secondary event, as already put forward by others (Berger et al. 1975). Interestingly, androgenic stimulation of early follicular growth was also suggested as a trigger for disturbed folliculogenesis (Hughesdon 1982). No structural change in poylcystic ovaries has been invariably reported in association with one definite biochemical finding, clinical sign or symptom of PCOS. In the present thesis, discussing some of the histological reports on polycystic ovaries was of interest for two main reasons. Firstly, as an absolute pre-requisite for sonography being used to assess ovarian structure in women with irregular menstrual cycles. Secondly, because it showed that investigators have pointed to the same type of changes. Structural changes of polycystic ovaries might be viewed, however, as the result of a longstanding disease, sometimes beginning at puberty.

4.5 Clinical features

SIGNS AND SYMPTOMS ASSOCIATED TO PCOS (G & A 1963)

	Incidence (in % of 1079 cases)	
Symptoms	Mean	Range
Infertility	74	35 - 94
Hirsutism	69	17 - 83
Amenorrhea	51	15 - 77
Obesity	41	16 - 49
Functional bleeding	29	6 - 65
Dysmenorrhea	23	------
Virilization	21	0 - 28

A most extensive review of clinical features of PCOS was published by Goldzieher and Axelrod in 1963. In this classical work, observations obtained from 187 reports with a total of 1079 cases were summarized. To the exception of alopecia and acanthosis nigricans, their observations cover all signs and symptoms widely accepted as associated to PCOS.

In addition, in 22% (0 - 71) a corpus luteum was observed at operation, 15% (12 - 40) disclosed a biphasic basal temperature curve, and 12% (7 - 28) of cases were regularly cycling. Reported signs probably reflect the field of interest of the investigator, and the index of suspicion for the disease. Variation in the frequency of any given symptom associated to polycystic ovaries is therefore impressive. This huge diversity of clinical pictures even led some people to doubt PCOS was a real entity, a true syndrome (Givens 1984; Moltz 1985). After the work of Axelrod and Goldzieher was published (1963), numerous publications have dealt with the clinical aspect of PCOS, illustrating the difficulty of establishing standard criteria. As different etiological models were progressively proposed (see above), further attempts were made to link clinical presentation to pathogenic factors, but no one association was found to be restrictive.

Very recently, two additional studies reported about large series of PCOS (Franks 1989; Conway et al. 1989). These studies are of special interest in the context of the present thesis, because both used detection of polycystic ovaries by (transabdominal) sonography as the only criterion for inclusion. A polycystic ovary was defined as one which contains, in one plane, at least 10 follicles measuring 2 to 8 mm in diameter, and an increased stroma echogenicity.

SIGNS AND SYMPTOMS ASSOCIATED WITH POLYCYSTIC OVARIES DIAGNOSED BY TRANSABDOMINAL SONOGRAPHY

	Incidence (in % of n)	
Symptoms	Franks (n=300)	Conway (n=556)
Infertility	42	29
Hirsutism	64	61
Amenorrhea	28	26
Oligomenorrhea	52	45
Obesity	35	35
Dysmenorrhea	14	--
Virilization	--	--
Acne	27	24
Alopecia	3	8
Acanthosis Nigricans	0.6	2

As compared to Axelrod and Goldzieher data (1963), hirsutism, infertility, and cycle disturbances still rank first. However, incidences differed. This may represent interobserver variations, or possibly different criteria of observation. For example, ways to perform hair grading differed because Axelrod and Goldzieher did not employ the now widely used Ferriman & Gallwey Score (1961). Moreover, obesity may be defined as a body mass index (BMI; Quetelet Index) above 25 kg/m^2 (Thomas and McKay 1976); this strict limit may not have been applied by all authors. Except for the incidence of infertility, there was a high degree of similarity between the two ultrasound based study populations.

It has been proposed to classify PCOS in type I, alleging an ovarian origin, and type II of probable adrenal origin (Raj et al. 1977). Others proposed that any patient with findings within the spectrum of signs and symptoms tabulated above may be diagnosed as having polycystic ovarian disease, whereas the eponym "Stein-Leventhal syndrome" would be reserved for the classical case with amenorrhea, anovulation, hirsutism and bilaterally enlarged ovaries (Thompson and Taymor 1980). Since Stein observed only 108 cases corresponding to his initial description in a thirty years period, the latter proposition may be reasonable. Later, Young and Goldzieher (1988) mentioned that it had become increasingly difficult to isolate diagnostic entities dealing with conditions that have hyper-androgenism "at or near their epicenter". In the very context of PCOS, absence of consensus for terminology, description, and evaluation, is rule rather than exception. As long as general agreement will not have been reached a considerable amount of energy will be wasted, be it in research or in treatment regimens. One attempt in the present thesis will be to establish a classification

primarily based on the structural aspect of the ovary, as assessed by transvaginal sonography.

Two additional issues which may be of clinical importance, but lie beyond the scope of this thesis, will be briefly mentioned. Firstly, Jackson and Dockerty (1957) reported 43 cases of polycystic ovaries in association with endomentrial carcinoma. Furthermore, they observed no difference in clinical features between women with or without carcinoma. Chronic anovulation may be viewed as a state of unopposed secretion of estrogen by the ovaries, and the endometrium was found as the only site at increased risk of neoplasia under this condition (Coulam et al. 1983). Secondly, persistent estrogenic stimulation in the absence of sufficient cyclic progesterone secretion may affect the incidence of mammary carcinoma (Sherman et al. 1974).

4.6 Endocrine features

Biochemical features of PCOS, like clinical symptoms, vary in frequency in patient groups diagnosed as PCOS. Criteria generally used for diagnosis are as follows: 1) high LH levels, 2) LH to FSH ratio above 2 or 3 (Lobo et al. 1981), 3) elevated androgen levels (such as T, DHEAS, AD, 4) raised T to SHBG ratio, described as the free androgen index (FAI). The predictive value, for the diagnosis of PCOS, of some of these endocrine estimates has recently been evaluated, and FAI gave the best overall accuracy with 94%, AD gave 74%, T 71%, and LH 69%, (Fox et al. 1991). FAI together with LH was the best combination (97%). A total of 856 women reported in studies by Franks (1989) and by Conway and colleagues (1989) has been screened for gonadotropins, PRL, T, and AD levels in peripheral blood. Compiled results are displayed here under, and demonstrate that LH and T values are significantly different from control groups (not shown) in the majority of cases.

ENDOCRINE DATA FROM 856 WOMEN WITH POLYCYSTIC OVARIES AS DIAGNOSED BY ULTRASOUND

	Franks (n=300)#	Conway (n=556)##
LH (IU/L) **	13.6 ± 8.8*	8.7 (8.2 - 9.2)*
FSH (IU/L)	4.3 ± 1.9	4.3 (4.1 - 4.4)
PRL (mU/L)	381.0 ± 675*	284.0 (265 - 305)
T (nmol/L)	3.0 ± 1.3*	2.5 (2.4 - 2.6)*
AD (nmol/L)	9.9 ± 4.2	---

mean ± SD ## mean (95% confidence interval)
(*) indicates values significantly higher ($P<0.05$) as compared to controls.
** By using RIA.

4.7 Incidence and prevalence

Because of the heterogenous mode of presentation of the syndrome, prevalence of PCOS has proven difficult to assess. Polycystic ovaries have been found in non-hirsute women who had conceived (Jeffcoate 1963), and in regularly cycling but infertile women (Vatukaitis 1983). Values of the incidence of polycystic ovaries based on pathological examinations range between 1.4% (McGoogan 1954) and 4.3% (Breteche 1952). In what proportion women reported as having morphologically proven polycystic ovaries were presenting signs or symptoms of PCOS is not known.

In order to characterize the incidence of polycystic ovaries in women with normal menstrual cycles, Polson and co-workers (1988) performed pelvic transabdominal sonography in 157 volunteers. 33 of them exhibited polycystic ovaries, yielding an incidence of 22%. This is strikingly high as compared to morphological data gathered from literature. One explanation may be that these were unselected volunteers who "found themselves to be normal", among which 28 (18%) had irregular cycles, and 7 (4%) were amenorrheic. Indeed, only 8 out of the 33 women with polycystic ovaries had regular cycles, and 6 out these 8 women were hirsute.

General population (i.e. including men and children) based calculations of the prevalence of PCOS presenting with infertility and cycle disturbances were published by Hull (1989). The estimated annual incidence of infertility associated with oligo-amenorrhea was 247 per million (= 0.025%) of the general population, 79 per million with amenorrhea and 168 per million with oligomenorrhea. 37% of the amenorrheic cases, and 90% of the oligomenorrheic cases were associated with PCOS. These results are in keeping with reported sonographic identification of polycystic ovaries in 32% of 100 women with amenorrhea, and 87% of 75 women with oligomenorrhea (Franks 1989). Moreover, the prevalence of ultrasound-diagnosed polycystic ovaries among first-degree relatives of women with symptomatic PCOS has also been investigated (Hague et al. 1988). A familial tendency was disclosed in 92% of the families studied. This latter observation may bring additional support for a genetic basis of the disease.

Chapter 5

A model to study the effect of androgens on granulosa cell function and ovarian follicle growth in the human

5.1 Introductory remarks

The primary lesion site has not been determined in PCOS. However, there is little doubt that ovarian androgens are produced in excess, and may initiate or perpetuate an anovulatory state. Androgen production in the thecal-interstitial compartment is regulated by LH, and modulation by paracrine factors (Hsueh et al. 1987; Cara and Rosenfield 1988), and possibly also by insulin (Barbieri et al. 1986; Poretsky and Kalin 1987; Poretsky et al. 1988) does occur. Precise inter-relationships are not yet elucidated, and ovarian tissue obtained from human polycystic ovaries is necessary to further investigate these issues in vitro. Next to cell cultures, immunocytochemistry and molecular biology techniques could be used to assess mechanisms of follicular cells dysregulation. However, ovarian wedge resection has been abandoned, and tissue is not readily available. Furthermore, a model for in vivo studies, in which conditions linked to PCOS could be created, would be ideal.

5.2 Animal models for the study of polycystic ovaries

To work out hypotheses related to the pathogenesis of polycystic changes, attempts have been made to set up animal models. Browman (1937) described that constant illumination can disturb the oestrus cycle in the rodent. Under these experimental conditions, a decreased pituitary LH content was reported (Maric et al. 1965), and typical morphological polycystic changes in the rat could be induced (Singh 1969). But the proestrus LH surge was systematically suppressed (Daane and Parlow 1971), and E2 could not elicit a LH surge (Mennin and Gorski 1975). Hence, it was considered that a model alterating feed-back at the hypothalamo-pituitary level did not meet the criteria to represent PCOS, and the project was abandoned. The nymphomanic cow presents with androgen excess and polycystic ovaries (Garm 1949; Short 1962b), but use of these animals was unpractical. Therefore investigations with the cow model were discontinued. Barraclough (1961) induced ovulatory failure and formation of polycystic ovaries in newborn female rats by administrating testosterone propionate. But this effect was linked to masculinization of the hypothalamus and consequent tonic male pattern of LH secretion (Barraclough and Gorski 1961). Again, this model was

discarded. The use of rats, androgen-sterilized in their neonatal period, has been considered as a suitable model by some authors (Goldzieher and Axelrod 1963), but questioned by others (Mahesh et al. 1987). Doubts were raised because exposure to androgens in the fetal and neonatal period are very different in rodents as compared to the human (Mahesh et al. 1987). Since hypothalamic, pituitary, adrenal and ovarian fuctions have all been cited as putative sources of polycystic changes, Mahesh and co-workers advocated that any animal model should absolutely present with normal function of these organs. Based on their common work with Roy (1962), in which AD and DHEA administration induced polycystic ovarian changes, they set up a model in immature 27 day old female rats. However, androgen administration depressed LH levels and FSH was increased in this model (Knudsen et al. 1975), whereas it is the opposite in women with polycystic ovaries. More recently, Faiman and colleagues (1988) tried to develop a rhesus monkey model for the study of PCOS. They implanted subcutaneously T in 25 animals, to achieve serum T concentrations between 2.8 and 3.9 nmol/L for a 13 to 16 months period. Menstrual cycle frequency was significantly reduced, but morphological examination revealed no differences in ovarian cortex thickness, or in number, size and proportion of the healthy and atretic follicles, as compared to a control group in which serum T was 0.8 nmol/L. It was hypothesized that exposure period to androgens was to small for inducing ovarian structural transformations. It may also be that the concentration of T was not high enough to induce marked changes.

Thus, to date, no convincing animal model for the study of polycystic ovaries, or of PCOS as a whole, has been established. In addition, two important considerations should be made which concern rodent models. Firstly, LH is an important hormone for ovulation in the human, while in the rat LH and prolactin are both required. Secondly, DHEAS levels are increased in several documented cases of polycystic ovaries in the human, while rat adrenals do not contain 17-α hydroxylase, consequently 17-OH PROG and androgens can not be synthesized.

Considering species differences, the concept of an animal model was purely abandoned in the present thesis. The work presented in this chapter was performed to find and characterize a human model for the study of the pathophysiology of polycystic ovaries.

5.3 Ovarian morphology in long term androgen treated female to male transsexuals. A human model for the study of polycystic ovarian syndrome?

Introduction

The pathogenesis of polycystic ovarian syndrome (PCOS), the most common ovarian disorder in women in their reproductive period, remains highly controversial (Franks 1989). However, clinical and biochemical signs of hyperandrogenism have been reported with constancy in PCOS (Greenblatt and Mahesh 1976; Barnes and Rosenfield 1989), and androgens are likely to play a pivotal role in initiating or

perpetuating this pathologic condition. Consecutive to defective aromatisation, accumulation of precursor androgens has been demonstrated in ovarian tissue from polycystic ovaries (Short and London 1961; Axelrod and Goldzieher 1962; Mahesh and Greenblatt 1962). Erickson and Yen (1984) further emphasised that in polycystic ovaries, continuous exposure of follicles to elevated androgen concentrations was a consequence of augmented theca-interstitial cell activity, in response to high luteinising hormone (LH) serum levels. Under normal conditions androgens have been shown to be both inhibitory and stimulatory in the control of follicle development (for review, see Daniel and Armstrong 1986). Thus, further studies at the ovarian tissue level are mandatory. However, availability of human ovaries is limited since wedge resection is no longer the first choice in treatment of patients affected by PCOS.

There is a unique and ethically acceptable possibility to explore the effects of androgens on human female ovarian function. Women with transsexualism who ask for gender reassignment receive long-term androgen treatment before undergoing surgery. This type of medication has been reported to induce polycystic changes in the ovaries (Miller et al. 1986; Amirikia et al. 1986; Futterweit and Deligdisch 1986; Spinder et al. 1989). Use of ovarian tissue obtained from these women implies, as a first step, detailed evaluation of the effects of androgens on ovarian morphology. Secondly, comparison with widely accepted histological criteria for the diagnosis of polycystic ovaries will be possible (Hughesdon 1982). Accordingly, the present study was designed to thoroughly assess histological changes in ovaries from female to male transsexuals (TSX) after long term exposure to androgens, in order to appraise their possible use as a human model for studies of PCOS.

Materials and methods

The study protocol was approved by the Ethics Review Committee of the Erasmus University, and consent was obtained from each patient. 17 women with transsexualism underwent hysterectomy and bilateral oophorectomy. Mean age at time of surgery was 25 years (range 18 to 35). Menstrual cycles were regular before androgen therapy was started in all but 4 women. Testosterone (T) treatment was given for a mean period of 21 months (range 11 to 72) before surgery took place. 13 women with regular menstrual cycles, on average 29 years old (range 27 to 39) underwent surgery for nonendocrine reasons and provided 14 ovaries which served as controls.

Mainly two types of T were administered before surgery, Sustanon (Organon, Oss, The Netherlands), of which 250 mg once each second week were given intramuscular for a mean period of 25 months (range 12 to 72) to 10 women, and Andriol (Organon, Oss, The Netherlands) which was given orally, 120-160 mg/day for a mean period of 15 months (range 12 to 21) to 6 women. Dihydro-testosterone, 125 mg/day (VU Hospital Pharmacy, gel preparation) was given to one woman, for a 11 months period.

In the operating room, both ovaries were measured in 3 dimensions (longitudinal [A], transverse [B], and antero-posterior [C]) immediately after oophorectomy had

taken place, and ovarian volume was calculated according to the formula ½ x A x B x C. Transported fresh to the laboratory, ovaries were observed macroscopically, focussing on size. After bisecting the ovary, attention was paid to the appearance of the cortex and presence of follicle cysts. 5 ovaries had been frozen for transportation at -70° in liquid nitrogen, and were not included in this study. Histological sections were made longitudinally throughout the largest diameter of the ovaries. Paraffin sections were stained with Haematoxylin Azophloxin (H & A), and with Masson's trichrome stain to further facilitate the exact measurement of the collagenised cortex and counting of atretic follicles. Microscopically, the largest section of each ovary was chosen to assess: 1) collagenisation and thickness of the cortex, 2) presence of primordial follicles, term used in presence of a single layer of flat granulosa cells, 3) number and size of healthy antral follicles, defined as antral follicles bordered by 2 or more layers of cubical granulosa cells, 4) number and size of cystic atretic follicles, defined as cavitary follicles with partly collagenised walls, loosing or having lost their granulosa cells, 5) number and size of atretic follicles, defined as follicles having lost their granulosa cell content, together with shape being lost and walls becoming completely collagenised, 6) stromal hyperplasia, 7) stromal luteinisation, and 8) luteinised theca interna cells. All follicles were defined irrespective of size. Cortex and follicle size measurements were performed using an eyepiece micrometer. Cortex was measured in 3 randomly chosen sites, and the mean of the 3 measurements was registred as thickness value. Mean diameters of antral and cystic follicles were assessed from the transverse and antero-posterior diameters. Sizes of atretic follicles were also estimated from these two diameters, from the exterior limit of one wall to the opposite one. The method employed did not represent true follicle diameters, and no attempt was made to estimate follicle size from measurement of profile distribution. Nevertheless, results gave a broad indication of follicle sizes in each category.

Measurements were always performed by the same observers together (S.C. and T.D.P.). TSX and control ovaries were assessed in a similar manner. Since ovarian volume, stroma, and cortex thickness, and follicle number measurements were strongly correlated between both ovaries, mean values per patient were calculated in all cases the 2 ovaries were available. Calculated values were used for comparison between the TSX and the control group, using the Mann-Whitney's Test. Measured follicle diameters were compared between both groups, taking account of patient differences, using analysis of variance. This was performed after logarithmic transformation to remove the skewness of distributions. The difference in stroma appearance was assessed using the chi-square test. Data are given as mean $\pm$ standard deviation, unless otherwise indicated. P values are two-sided with 0.05 being the limit of statistical significance.

Results

29 ovaries were obtained from 17 female to male TSX, who all displayed a male phenotype and had become amenorrheic after a period of androgen treatment

ranging from 12 to 72 months (mean 21 months). 14 control ovaries were procured by 13 regularly menstruating women.

Macroscopically, 11 out of 29 ovaries appeared grossly enlarged, giving the impression the number of follicle cysts was elevated. When calculated, mean ovarian volume in TSX was 7.6 ± 3.6 ml, which was notably different ($P<0.001$) from the volume estimated in controls, equivalent to 2.7 ± 1.4 ml. A paler, greyish color was apparent in 5 out of the 11 above mentioned ovaries. Histopathological findings in TSX ovaries are displayed in Table 1, and observations made in normal ovaries are exposed in Table 2. Microscopically, all TSX ovaries but one exhibited a thickened collagenised cortex, with a mean thickness of 817 ± 300 µm (Fig. 1). This was significantly larger ($P<0.001$) than in controls, in which a mean cortex thickness of 241 ± 85 µm was observed. The transition between cortex and sub-cortical stroma was by far less distinct in normal ovaries as compared to TSX ovaries.

Table 1. Histological characteristics in 29 ovaries from 17 female to male transsexuals after long-term androgen exposure

		Ovary			Follicles					
					Healthy antral		Cystic atretic		Atretic	
Patient no.	Side	Volume (ml)	Cortex (µm)	Stroma	No.	Mean size (µm)	No.	Mean size (µm)	No.	Mean size (µm)
1	L	1.6	800	c	1	200	11	2600	20	494
2	R	1.9	850	b	1	630	3	4500	20	402
3	L	7.4	800	c	0	—	8	3925	25	360
4	L	6.2	1400	c	1	200	2	3900	11	355
5	R	17.2	250	b	1	200	9	2355	23	1289
	L	11.9	200	b	2	162	11	2473	9	978
6	R	10.9	800	c	2	450	13	2100	3	692
	L	13.1	600	c	2	237	18	2171	3	1067
7	R	9.5	1300	b	0	—	4	2400	3	408
	L	13.4	1200	b	0	—	7	2443	4	560
8	R	4.6	1200	b	1	100	8	2662	19	574
9	R	8.4	600	b	0	—	5	2620	17	768
	L	12.4	1600	b	1	500	4	1600	13	406
10	R	3.6	500	b	0	—	1	750	3	250
	L	5.4	760	b	0	—	1	550	2	138
11	R	9.4	400	b	0	—	12	2192	3	1267
	L	8.4	800	b	2	290	16	2137	2	275
12	R	7.5	300	c	2	650	11	1518	16	425
	L	5.4	650	b	3	500	11	1914	15	480
13	R	11.2	700	b	0	—	17	1205	20	386
	L	8.0	600	b	2	262	10	2030	31	330
14	R	7.6	600	c	1	425	11	1409	50	245
	L	6.9	600	c	2	562	19	1728	40	134
15	R	9.8	1100	c	2	75	6	3210	34	515
	L	10.9	1100	c	2	87	13	2060	20	590
16	R	4.6	800	c	0	—	4	944	24	396
	L	5.7	800	c	0	—	10	770	18	170
17	R	NA	800	b	0	—	10	1015	20	525
	L	NA	600	b	1	550	17	2159	14	514

b = hyperplasia: c = hyperplasia + clusters of luteinized cells: NA = not available.

Table 2. Histological characteristics in 14 ovaries from 13 women with regular menstrual cycles

		Ovary			Follicles					
					Healthy antral		Cystic atretic		Atretic	
Case no.	Side	Volume (ml)	Cortex (μm)	Stroma	No.	Mean size (μm)	No.	Mean size (μm)	No.	Mean size (μm)
1	L	6.0	400	a	1	175	3	1200	2	750
2	R	2.5	350	a	0	—	3	2333	7	300
3	L	3.4	180	a	3	327	5	1500	4	643
4	R	1.7	100	a	2	2200	2	5500	15	489
	L	1.5	200	a	1	200	1	4400	11	400
5	L	3.7	250	a	0	—	5	2760	5	620
6	L	3.3	200	a	0	—	3	3333	1	2200
7	R	2.4	250	a	0	—	6	2350	4	1875
8	R	0.8	300	a	1	220	2	2250	3	1000
9	L	1.3	250	a	0	—	6	2317	3	1023
10	L	1.8	300	a	2	340	2	2350	8	500
11	R	2.4	100	a	3	487	7	3471	1	1000
12	R	4.3	150	a	0	—	6	3383	3	633
13	R	2.5	250	a	2	300	9	2064	14	385

a = normal cellularity.

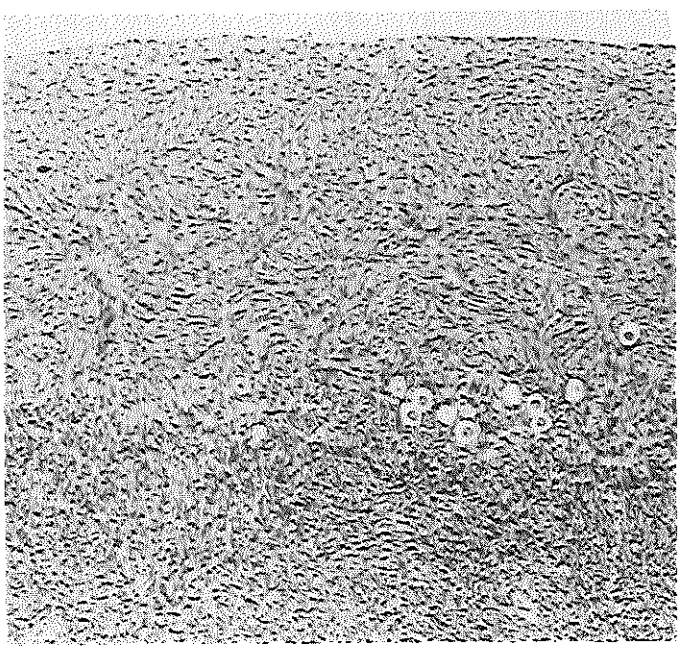


Figure 1. Thickened collaganized tunica albuginea in ovary of transsexual. H & E. x 75.

In every control ovary, and in all TSX ovaries except one, primordial follicles were observed. The total number of healthy antral, cystic atretic, and atretic follicles together was 27 ± 13 in TSX, and was higher ($P<0.001$) than the total number observed in controls, which corresponded to 11 ± 5 follicles. Healthy antral follicles were seen in 18 out of 29 TSX ovaries, never more than 2 follicles in one section, and their mean size was 313 ± 180 µm. Healthy antral follicles were seen in 8 out of 14 normal ovaries, never more than 3 in one section, and their mean size was 535 ± 494 µm. Between TSX and controls, there was no significant difference in number or size for these healthy follicles. However, the small number of follicles observed hampered statistical evaluation.

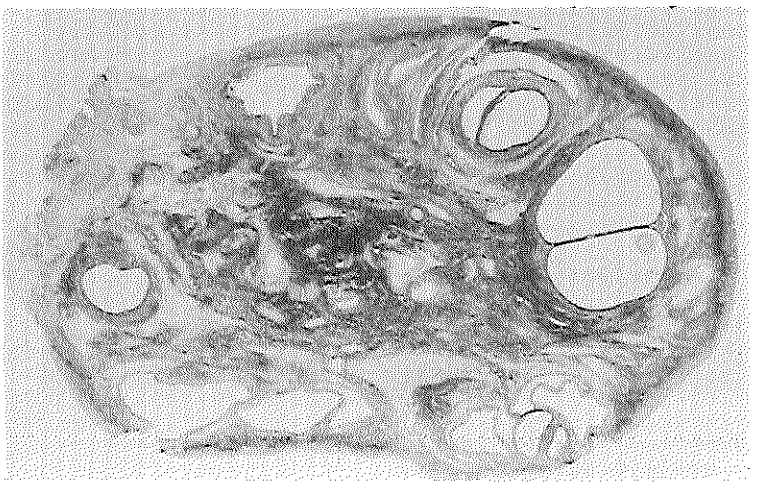


Figure 2. Section through ovary of transsexual, showing multiple cystic atretic follicles. H & E. x 4.

Multiple cystic atretic follicles were present in all TSX ovaries (Fig. 2), on average 9 ± 5 follicles in the observed section. Their mean size was 2297 ± 861 µm. In control ovaries, 4 ± 2 cystic atretic follicles were observed on average, displaying a mean size of 2700 ± 1023 µm. The difference in mean number of cystic atretic follicles was high ($P<0.01$) between the 2 groups, whereas mean sizes were not significantly different. Theca interna hyperplasia was observed in all follicle cyst walls of TSX ovaries without exception (Fig. 3), but was seen only one time in follicle cyst walls of control ovaries. In addition, many theca interna cells from TSX cystic atretic follicles were luteinised, which was the case only once in follicle cysts from control ovaries. A mean number of 17 ± 11 atretic follicles were counted in all TSX ovaries, on average sized 547 ± 252 µm, as compared to a mean of 5 ± 4 atretic follicles in control women, measuring on average 875 ± 570 µm. Again, the number of atretic follicles was much higher ($P<0.005$) in TSX than in controls, while differences in size did not reach the level of statistical significance.

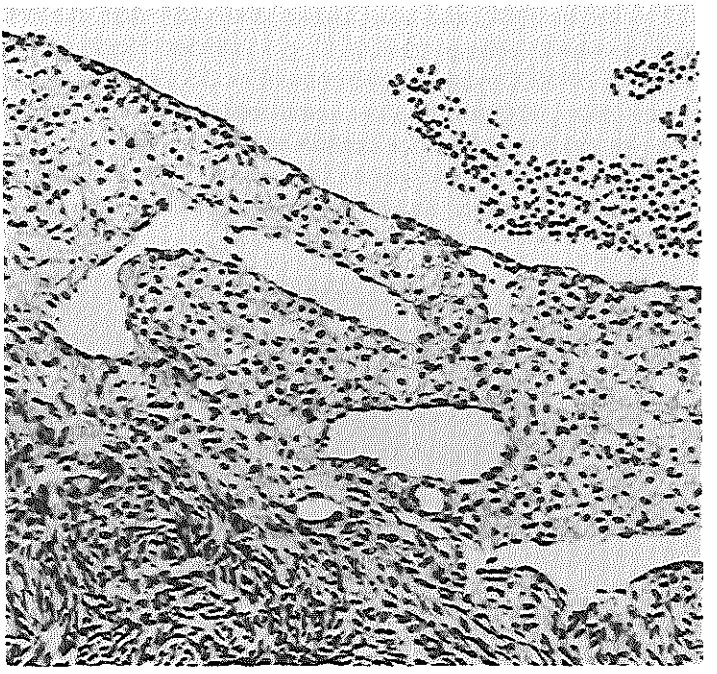


Figure 3. Theca interna hyperplasia with luteinization in the wall of a cystic atretic follicle. H & E. × 380.

In all TSX ovaries, diffuse stromal hyperplasia was present, and isolated luteinised cells could be observed in the stroma. Moreover, in 12 TSX ovaries, luteinised stromal cells were organised in clusters (Fig. 4). Control ovaries did exhibit neither stromal hyperplasia, nor stromal luteinisation. Accordingly, the difference in stroma appeareance between TSX and controls was significant ($P<0.001$).

Discussion

Opportunities to describe the effects of androgens on human ovarian tissues have been scarce. Among the authors who reported on these effects (Miller et al. 1986; Amirikia et al. 1986; Futterweit and Deligdisch 1986; Spinder et al. 1989), only Amirikia et al. published quantified observations. The present study provides detailed information about histological changes in human ovaries consecutive to exposure to exogenous androgens for a mean period of 21 months.

The TSX ovaries we observed were on average 2.5 times larger than controls. Volumes above 8.0 ml - the same range indicated by Amirikia and colleagues (1986) -

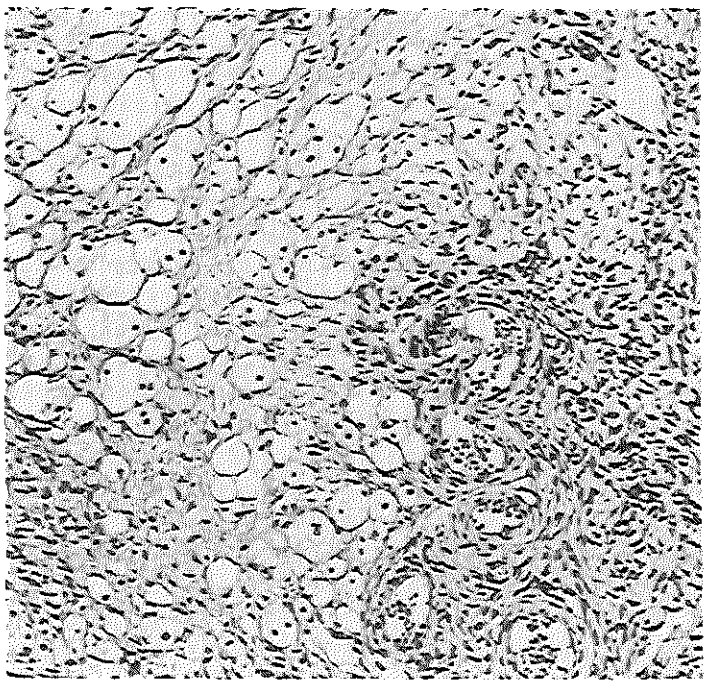


Figure 4. Clusters of luteinized cells in a hyperplastic ovarian stroma. H & E. × 380.

were disclosed in 14 out of 29 ovaries. One fourth of the TSX ovaries observed by Futterweit and Degligdisch (1986) were considered as enlarged, whereas no size difference as compared to controls was reported by Spinder et al. (1989). The high number of cystic follicles together with the augmented stromal cellularity observed in the present study, probably fostered the increase in ovarian volume. A very thick cortex may not be negligible in volume calculation, but association between a large ovary and a broad cortex was not systematic in our study. Hughesdon mentioned that a larger size of polycystic ovaries could be related to a longer symptomatology period (1982). Patients from the 3 above mentioned studies with TSX (Amirikia et al. 1986; Futterweit and Deligdisch 1986; Spinder et al. 1989) received on average T for 35, 36 and 18 months, respectively. In view of this, dissimilar reports on ovarian size can not be attributed to the duration of androgen treatment. Polycystic ovaries were originally reported as being from 2 to 4 times the normal size (Stein and Leventhal 1935). This ovarian volume increment was corroborated by most authors (Hughesdon 1982; Plate 1958; Goldzieher and Green 1962; Vaughan Jones 1962), but not by all of them (Smith et al. 1965).

It has been shown shown that cortex was 4 times thicker in TSX ovaries than

in controls (Amirikia et al. 1986). A collagenised cortex was further reported in TSX ovaries (Futterweit and Deligdisch 1986; Spinder et al. 1989), in respectively 96% and 68% of the cases. The 93 % incidence observed in the present study, combined with a three-fold thicker cortex as compared to controls, provide additional evidence that high levels of androgens can be related to a marked increase of cortex collagenisation. In treatment of patients affected by PCOS, use of ovarian wedge resection and laparoscopic ovarian cautery have brought indirect evidence of this relationship, since both procedures decreased androstenedione and testosterone serum levels (Mahesh et al. 1978; Sakata et al. 1990). Unlike normal ovaries, the transition between cortex and subadjacent tissues was abrupt. This latter observation has also been reported in polycystic ovaries (Hughesdon 1982; Goldzieher and Green 1962).

It is noteworthy that primordial and healthy tertiary follicles appeared to be equally present in TSX and in controls. Follicle count was performed with great care by Hughesdon (1982), and the overall average of primordial follicles also seemed to be much the same in normals and in polycystic ovaries. The same author claimed that the number of all forms of ripening follicles was almost doubled as compared to controls. This increase was more marked with regard to the smaller tertiary forms. Comparison with our data is difficult since he considered the terms antral, tertiary and cystic as synonymous whereas we discriminated between healthy antral and cystic follicles in the present study. Moreover, the number of healthy follicles we observed was to small for meaningful comparison between groups. However, in our study, all forms of follicles taken together - healthy antral, cystic atretic, and atretic - were also very much increased in number in TSX, as compared to controls. The number of cystic follicles alone was twice higher in TSX than in controls, and it may be inferred that this increase is consistent with the abnormally high number of cystic follicles generally observed in polycystic ovaries. One may therefore speculate that cystic conversion rate is accelerated under exposure to high androgen levels. In this regard, we consider that three and a half times more atretic follicles in TSX ovaries than in normal ovaries comes as no surprise. Besides, since in TSX sizes of cystic and atretic follicles were not different from in controls, this may indicate that mechanisms involved in cystic transformation and in atresia remain merely similar under both normal and hyperandrogenic condition.

Two structural differences were observed between cystic follicles of TSX and normal ovaries. Firstly, theca interna hyperplasia was constant in the ovaries we looked at. It has been asserted that theca hyperplasia, even if not considered a specific criterion for polycystic ovaries, remained an adequate histological basis for diagnosing the disease (Vaughan Jones 1962). Moreover, theca interna layer was thicker in a well-documented series of 39 patients with polycystic ovaries (Goldzieher and Green 1962). Thus, it appears that androgens either of endogenous or of exogenous origin may be capable of inducing such changes. Secondly, all TSX cystic follicles we examined displayed a number of luteinised theca cells. This is in partial agreement with other studies in wich a lower incidence was disclosed (Futterweit and Deligdisch 1986; Spinder et al. 1989). These luteinised cells exhibited a more abundant cytoplasm

without being fully vacuolised, and vesicular nuclei with well limited nucleoli. A luteinised theca cell layer has been well described in polycystic ovaries (Plate 1958; Vaughan Jones 1962) . However, it has also been claimed that completely luteinised theca cells were not a feature of polycystic ovaries (Goldzieher & Green 1962). The appreciation between partial and complete luteinisation of a cell remains subjective. Nevertheless, observations in TSX and in polycystic ovaries may indicate that under both conditions, a higher than normal androgenic effect proved to be capable of inducing theca interna hyperplasia and luteinisation.

Stromal hyperplasia was a uniform finding in the present investigation, and related in more than 80 % of the cases in similar studies performed by Futterweit and Degligdisch (1986), and by Spinder and co-workers (1989). At the same time, they yielded a 27% incidence of stromal luteinisation. With the inclusion of isolated luteinised stromal cells we would have reported an incidence of 100 %, but sticking to a cluster organisation of these cells -as the above mentioned authors probably did - we reached an incidence of 35 %. In polycystic ovaries, this kind of luteinised cells foci in stroma have been described (Hughesdon 1982; Stein & Leventhal 1935). Although stromal hyperplasia and luteinisation were not constantly portrayed, their occurence in polycystic ovaries is unequivocal (Goldzieher 1981). These changes may be consecutive to increased LH stimulation of the thecal and stromal compartments, and ensuing augmented androgen production (Erickson and Yen 1984). On the basis of our observations, one would be tempted to speculate that this "trophic" effect is produced by administration of exogenous androgens. Interestingly in the case of TSX, the LH signal to theca cells is low as a result of the negative feedback action of androgens on the hypothalamo-pituitary axis (data not shown). This might indicate that androgens alone, independently from peripheral LH levels, are capable to induce polycystic changes. Accordingly, any condition susceptible to increase androgen concentration might lead to polycystic changes in the ovaries.

The site of the primary lesion is not known in PCOS. However, many women present with manifestations of hyperandrogenism, and histological changes characteristic of polycystic ovaries can be found when androgen overproduction is either intra-ovarian or extra-ovarian in origin (Dunaif et al. 1984; Lobo 1984). In addition, using transvaginal sonography, we have shown that ovarian follicle number and stroma echogenicity are correlated to serum androgens (Pache et al. 1991). Attempts to produce a picture resembling to human polycystic ovaries failed in the non-human primate (Faiman et al. 1988), and availability of a human model would suppress speculations related to species differences. We therefore reasoned that the validation of a human model would be of help for further investigations.

Our study suggests that the long term action of increased androgen levels adversely affects follicle growth in human ovaries. The observed histological changes we reported are in keeping with descriptions of polycystic ovaries by several authors. Moreover, our findings may provide direct evidence that the morphological changes are indeed due to androgen excess in polycystic ovaries. Furthermore, documentation of the striking histological similarity between polycystic and TSX ovaries provides a

unique possibility to study the role of androgens in the pathogenesis of PCOS in the human.

Summary

Descriptions of the effect of androgens on ovarian human tissues are exceptional. This opportunity was provided by 17 women with transsexualism - female to male transsexuals (TSX) - who had been given androgens for a mean period of 21 months before hystero-salpingo-oophorectomy took place. 29 ovaries from TSX and 14 control ovaries from 13 regularly cycling women were examined. As compared to controls, TSX ovaries were enlarged, displayed a 2-fold increase in cystic follicles, a 3.5-fold increase in atretic follicles; the ovarian cortex was collagenised and three times thicker. Theca interna hyperplasia and luteinisation were uniformly observed in TSX cystic follicles. Stromal hyperplasia was a constant finding in TSX ovaries, accompanied by clusters of luteinised stromal cells in 12 cases. Eventually, these findings met the histological criteria for the diagnosis of polycystic ovaries. These observations demonstrate that androgens alone may induce polycystic changes. The assumption that the role of androgens is pivotal at the follicular level - inducing follicle growth arrest and accelerating cystic changes - in the genesis of polycystic ovaries is reinforced.

Chapter 6

Ovarian follicular growth under abnormal conditions

6.1 Introductory remarks

Reports concerning follicle number, volume and stroma echogenicity of polycystic ovaries are characterised by their extreme variability (Swanson et al. 1981; Parisi et al. 1982; Hann et al. 1984; Parisi et al. 1984; Orsini et al. 1985; Adams et al. 1985; Adams et al. 1986; Tabbakh et al. 1986; Yeh et al. 1987; Conway et al. 1989; Obhrai et al. 1990; Fox et al. 1991). There is clearly a need to reappraise and validate norms. Up to now, transabdominal sonography has been exclusively employed. However, this method is hampered by the limited potential of visualising small follicles. Furthermore, a full bladder is required for optimal examination conditions, which may distort pelvis anatomy (Timor-Tritsch et al. 1988). Besides, bowel loops adjacent to the ovary may interfere with the sound beam, or produce a shadow effect which obscures ovarian tissue. Using transabdominal sonography, Hull recently stated that it was impossible to adequately determine the ovarian architecture in as much as 42% of cases (1989).

As compared to abdominal sonography, use of the transvaginal technique presents the definite advantages of improved resolution and visualisation (Yee et al. 1987; Coleman et al. 1988), and of better patient acceptance (Goldstein 1990). It is assumed that a more detailed description of the abnormal ovarian structure of polycystic ovaries may improve the accuracy and correlations with biochemical and clinical signs. Furthermore, it could be that follicle number or stroma echogenicity reflect the severity of the disease.

It was the goal of this chapter to perform a stepwise approach to the sonographic investigation of polycystic ovaries. Firstly, data collected from previous ultrasound reports on polycystic ovaries are presented. Secondly, the ability of transvaginal sonography to assess ovarian structure in women with clinical and biochemical signs of PCOS was investigated. Thirdly, ovaries of regularly cycling women and ovaries of women with PCOS signs and symptoms have been compared, in an attempt to define diagnostic criteria of polycystic ovaries via the transvaginal route.

6.2 Sonographic diagnosis of polycystic ovaries

In the last decade, sonography has become the method of choice to diagnose polycystic ovaries. However, the number of proposed sonographic criteria is almost equal to the number of investigators who published their observations (Fauser 1991a). This disparity is illustrated in table 1 on the next page, in which data harvested from the literature are presented. It has to be realized that almost all investigations have been performed by the transabdominal route, and that these results extend over a 10 year period. In all these years technology progressed, and differences between early and late reports may be due, at least in part, to better ultrasound equipment.

6.3 Transvaginal sonography and abnormal ovarian appearance in menstrual cycle disturbances

Introduction

Amongst ovarian disorders in women in the reproductive age, polycystic ovary syndrome (PCOS) is probably the most common (Vaitukaitis 1983). However, substantial controversy still surrounds definition and diagnosis of this syndrome (Franks 1989). Initial diagnostic criteria were determined by histological and clinical features (Stein and Leventhal 1935; Goldzieher and Axelrod 1963). More recently, distinct biochemical features, such as selectively increased luteinising hormone (LH) levels (Rebar et al. 1976)) and augmented androgen levels (Raj et al. 1978) were added to morphological criteria. Ultimately, the availability of non-invasive ultrasound technology, made it possible to look for polycystic changes in the ovaries directly (Swanson et al. 1981; Parisi et al. 1982).

There are, however, two major shortcomings in the sonographic diagnosis of polycystic ovaries. Firstly, the limited capability of visualizing small follicles using transabdominal ultrasound. This is even more pronounced in obesity, which is a common finding in PCOS. Secondly, the knowledge of the normal range of follicle numbers is limited. Both restrictions brought about many different definitions of polycystic ovaries (Hann et al. 1984, Parisi et al. 1984; Adams et al. 1985; Orsini et al. 1985, Tabbakh et al. 1986; Yeh et al. 1987; Polson et al. 1988). Recently high-frequency transvaginal sonography was used to assess follicle growth patterns in normally cycling women (Pache et al. 1990).

The present study was designed to characterize ovarian structural changes in women suffering from cycle disturbances and infertility. In addition, observed changes were correlated with clinical and biochemical features relevant to the diagnosis of PCOS. Repeated sonography examinations were performed to address possible dynamic changes in the ovaries.

DESCRIPTIONS OF POLYCYSTIC OVARIES IN TRANSABDOMINAL SONOGRAPHY STUDIES

		Ovarian sonography		
Author	Number of patients	Aspect	Follicle number and size	Comments
Swanson 1981	863	ovaries 6 - 30 ml (mean 12.5 ml) 5% (n=22) enlarged	6 - 16 2 - 6 mm	No indication when US was performed. 22/863 did show polycystic ovaries.
Parisi 1982	78 9/78	cystic appearance PCO markedly enlarged thickened structure	many mostly <10 mm	No indication when US was performed in the cycle. PCO histology in 4/9.
Hann 1984	28 PCO	8/28 "normal" size 71% (n=21) enlarged, on average 14 ml hypoechoic without cysts in 25% (n=7) PCO ovaries	multiple <10 mm in 39% (n=11)	No indication when US was performed. PCO histology in 12/28.
Parisi 1984	187 26/187 PCO	bilateral enlargement sometimes "polycystic margins"	many 5 mm 15 mm (in 2/26)	No cystic structure in some patients (5/26). US when ?
Orsini 1985	50 PCO 25 controls	enlarged in 16/50 14 + 7.3 ml (SD) in PCO 8 + 2.3 ml in controls if one >10 mm b)	<4 <9 mm a) or multiple all small b)	US within CD 1-7 a)= solid form b)= cystic form
Adams 1985	76 17 controls 55 PCO - 21 MFO	14.6 + 1.1 ml (SE) in PCO 6.4 + 0.4 ml in controls 8.0 + 0.8 ml in MFO	>10 2 - 8 mm =PCO or many 2 - 4 mm =PCO 6 - 10 4 - 10 mm =MFO	stroma normal in multifollicular (MFO) ovaries. US when ?
Adams 1986	173 107/173 PCO		criteria as above	26% of amenorreic 65% of oligomenorreic had PCO US in cycle days 1 - 9.
Tabbakh 1986	20 PCO	enlarged in 15/20 isoechoic (n=4) hypoechoic (n=11) hypoechoic (n=5) 0 mm	0 many <10mm or 1 >10 mm	echogenic "capsule" of the ovary in 18/20. US in early follicular phase.
Yeh 1987	104 patients 25 controls	70% PCO ovaries enlarged 13 controls with >10 follicles < 3 mm.	>5 5 - 8 mm	15 - 29 mm follicles in 14% of PCO. US in cycle days 1 - 11.
Polson 1988	257 "normal" volunteers	23% PCO (n=36/257) most PCO >10 ml	idem Adams	"normality" decision by volunteers
Conway 1989	556 PCO 23 controls		idem Adams	polycystic ovaries at the US were the unique criteria for inclusion
Obhrai 1990	86 PCO 29 controls	>10 2 - 8 mm	idem Adams	polycystic ovaries at the US were the unique criteria for inclusion
Hull 1990	not specified	increased stromal echogenicity	>10 2 - 8 mm	overlap volumes in random arrangement polycystic, normal, and multicystic ovaries
Fox 1991	65 PCO	increased stromal echogenicity	15 2 - 10 mm	transabdominal 3.5 MHz usally around the and/or transvaginal US ovarian cortex 7.5 MHz

US = ultrasound
MFO = multifollicular ovaries
PCO = polycystic ovaries

Materials and methods

Subjects

Thirty-seven women who consecutively attended our Infertility Clinic presenting with oligomenorrea ([n=17] mean cycle length 65.4 days, range 35 to 99) or amenorrea ([n=20] no vaginal bleeding for >6 months), were enrolled in the study. Underweight women (body mass indexes [BMI] below 19 kg/m^2) were not included in the study. Mean age was 27.3 years (range 18 to 41), and mean infertility duration was 3.7 years (range 1 to 12). The study was approved by the Ethics Review Committee of the Erasmus University, and each woman gave informed consent.

Study protocol

Starting at the third or fourth day of the menstrual cycle, or at random in case of amenorrea, ultrasound examinations were performed on three occasions, with a three to four days interval between every session. A 5.0 MHz vaginal probe (Orion, Philips Medical System, Eindhoven, The Netherlands) was used. All examinations were performed by the same observer (T.D.P.) who scanned the ovaries according to a method described and validated previously (Pache et al. 1990). Briefly, follicles were first counted by scanning each ovary from the inner to the outer margin in longitudinal cross-sections. Follicle size was determined from 2 dimensions (< 6.0 mm) or from 3 dimensions (≥ 6.0 mm). Ovarian stroma echogenicity was scored as normal (=1), moderately increased (=2), or frankly increased (=3). Longitudinal (A), antero-posterior (B) and transverse (C) dimensions of the ovary were recorded, and ovarian volume determined according to the formula ½ x (A x B x C).

In every woman, BMI expressed in kg/m^2 was calculated. Hair grading was assessed according to the Ferriman and Gallwey score ([F-G] Ferriman and Gallwey 1961).

Hormone estimations

Blood samples were obtained through venepuncture the same day the first sonographic examination was carried out. Serum was assayed for LH, follicle-stimulating hormone (FSH), testosterone (T) and sex hormone binding globuline (SHBG). Immunoreactive LH and FSH were determined as described previously (Fauser et al. 1990a), using a commercially available radio-immunometric (IRMA) kit (Medgenix, Fleurus, Belgium). T was determined by radioimmunoassays (RIA) as described by Verjans (Verjans et al. 1973). SHBG levels were measured by IRMA, supplied by DPC (Diagnostic Products Corp., Los Angeles CA, USA). In the first half of the follicular phase, reference values from our laboratoray range from 1 to 7 IU/L for FSH, 1 to 8 IU/L for LH, 0.5 to 3.0 nmol/L for T, and 20 to 120 nmol/L for SHBG.

Data analysis

In every woman, sonographic findings were compared between ovaries and between each of three examinations of the same ovary. Data harvested from the first sonographic examination were correlated with the BMI and F-G score, and with two biochemical markers, the free androgen index ([FAI = T x 100/SHBG] Eden et al. 1989) and LH/FSH ratio.

Statistical analysis was carried out by using the Friedman-Wilcoxon test for paired data. Correlation coefficients are Pearson's (r), in a single instance Spearman's (r_s). P=0.05 (two-sided) was considered the limit of statistical significance. Results are presented as mean and standard deviation (SD)

Results

Sonographic examinations were performed three times in 33 women, twice in three women and once in one woman. Mean BMI was 25.8 ± 5.7 kg/m^2, mean F-G score was 12.2 ± 7.1. Mean LH to FSH ratio was 1.6 ± 1.0, and mean FAI was 6.7 ± 4.7.

Results of sonographic findings in subsequent examinations are presented in Table I. No statistically significant differences could be established between the right and the left ovary for mean values of ovarian volume, stroma echogenicity, number of follicles and follicle size. For all these parameters, no statistically significant difference was found between serial examinations of the same ovary. The total mean number of follicles observed in both ovaries did not differ significantly in subsequent examinations.

Table I. Observations during subsequent sonography examinations—with three- or four-days intervals—in 37 women with cycle disturbances. Initial ultrasound was performed around cycle day 3 in oligomenorrhea and at random in amenorrhea. Values represent mean ± SD.

	Ultrasound 1 (n = 37)		Ultrasound 2 (n = 36)		Ultrasound 3 (n = 33)	
	Right ovary	Left ovary	Right ovary	Left ovary	Right ovary	Left ovary
Follicle number	10.0 ± 7.2	9.3 ± 6.1	10.4 ± 6.7	9.6 ± 6.5	10.5 ± 7.1	8.8 ± 6.1
Follicle size (mm)	4.2 ± 1.1	3.8 ± 0.7	4.5 ± 1.3	3.8 ± 0.9	4.1 ± 1.2	3.9 ± 1.0
Ovarian volume (ml)	10.3 ± 5.5	8.5 ± 5.0	10.9 ± 5.9	9.1 ± 5.4	10.9 ± 5.1	9.7 ± 5.4
Stroma echogenicity*	2.4 ± 0.6	2.4 ± 0.6	2.4 ± 0.6	2.4 ± 0.6	2.4 ± 0.5	2.4 ± 0.5
Maximal follicle number in any one ovary	12.5 ± 7.1		12.2 ± 7.2		12.2 ± 7.6	
Total follicle number in both ovaries	19.4 ± 10.9		19.9 ± 11.2		19.3 ± 10.9	

* Ovarian stroma echogenicity was scored from 1 to 3

Right and left ovarian volumes were strongly correlated in all ultrasound examinations ($r \geq 0.65$, $p<0.001$). The volume of the right ovary at the first examination strongly correlated with its volume at the second examination (r=0.88, $p<0.001$), and the volume of the right ovary at the second examination was correlated with its volume at the third examination (r=0.86, $p<0.001$). For the left ovary, similar correlations were established (respectively: r=0.86, $p<0.01$; and r=0.94, $p<0.001$).

At all three examinations, follicle number in the right ovary was correlated with right ovarian volume (respectively: r=0.76, r=0.78, r=0.78; all $p<0.001$), and in the left

ovary with left ovarian volume (respectively: r=0.54, r=0.58, r=0.49; all p<0.05). Mean size of ovarian follicles on the right side correlated well with mean size on the left side (r>0.70, p<0.001). No statistically significant correlation could be demonstrated between mean follicle size and follicle number.

At the first sonographic examination, total follicle number in both ovaries correlated well with ovarian stroma echogenicity (r_s=0.70, p<0.001), and total ovarian volume (r=0.74, p<0.001) (Fig.1). At the same time, total follicle number correlated

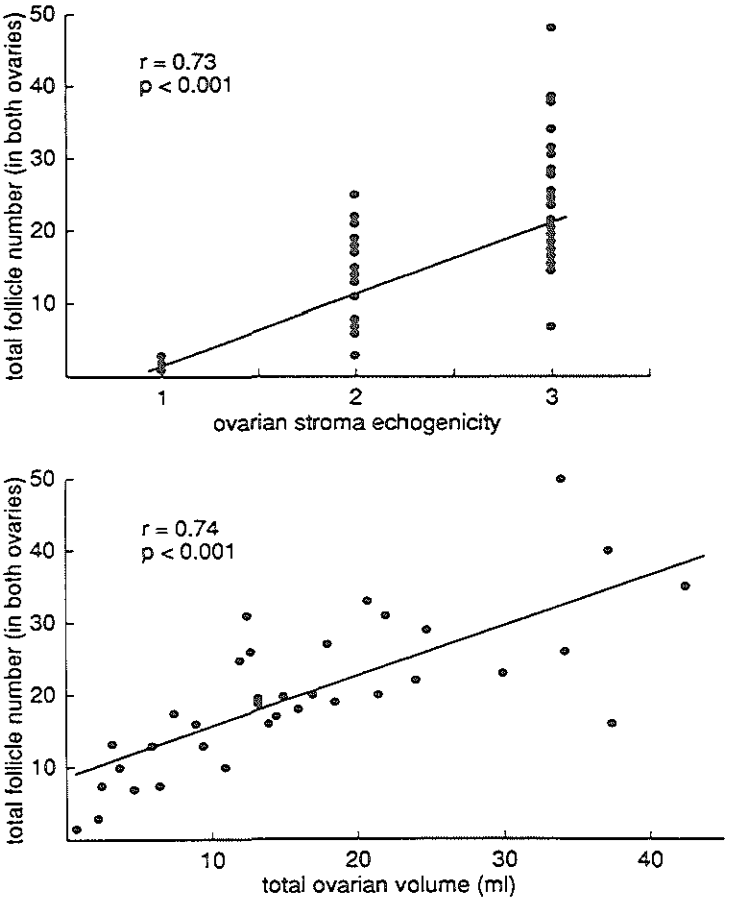


Figure 1. Correlations between total number of follicles and ovarian echogenicity, and total ovarian volume, in 37 women with abnormal menstrual cycles.

significantly with the FAI (r=0.37, p<0.05), and LH to FSH ratio (r=0.35, p<0.05) (Fig.2). A correlation between follicle number and BMI and F-G score could not be statistically demonstrated.

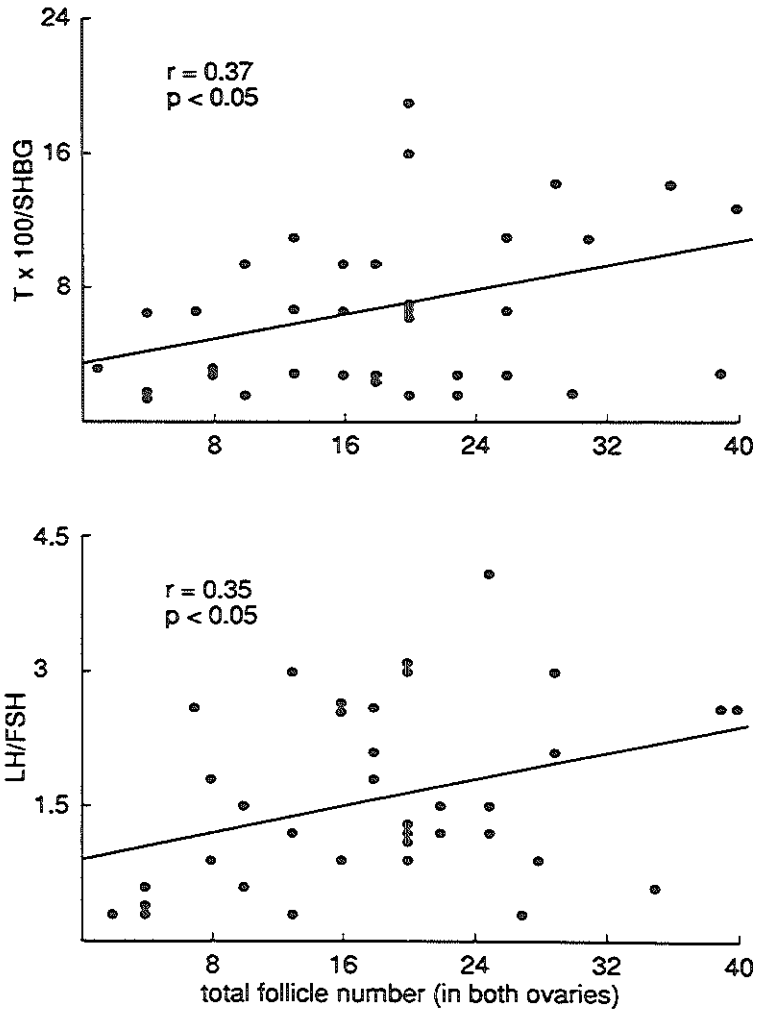


Figure 2. Correlations between total number of follicles, and the FAI (T × 100/SHBG ratio), and the LH to FSH ratio, in 37 women with abnormal menstrual cycles.

Discussion

Data discussed in this paper provide initial information related to sonographic changes in the ovaries of normal and overweight patients with cycle abnormalities. Attention was focussed on characterizing the appearance of the ovary, and possible correlations with clinical and biochemical features of PCOS.

Previous publications dealing with ovarian abnormalities in women with disturbed cycles offer controversial ultrasound definitions of polycystic ovaries (Hann et al. 1984; Orsini et al. 1985; Polson et al. 1985) (for review see Fauser 1990b). This may be attached to the use of transabdominal sonography which limits the ability to visualize small follicles, and to the lack of proper characterization of ovarian function under normal conditions.

Observations provided by the present study suggest that the mean number of follicles observed by transvaginal sonography in women with oligo or amenorrea (10.0 ± 7.2 on the right, and 9.3 ± 6.1 on the left side) is higher as compared with normal controls, in which a mean number of 6.2 ± 2.6 and 7.5 ± 2.8 follicles was observed in the right and the left ovary during the early follicular phase (Pache et al. 1990). Next to an abnormally high follicle number and an augmented ovarian volume, increased stroma is considered to be a classical morphological criterion for the diagnosis of polycystic ovaries (Hughesdon 1982). Our results show that follicle number is correlated with the estimated amount of stroma, and with ovarian volume. This suggests that the combination of an increased follicle number together with normal amounts of stroma, depicted as multicystic ovaries by Adams et al. (1985) is unlikely in normal weight or obese women. Mean ovarian volumes were higher than in normal women (for example, right and left ovaries of 10.2 ml and 8.4 ml, versus 4.7 ml and 5.4 ml as measured in early follicular phase of normal women [Pache et al. 1990]). Moreover, a correlation was found between total follicle number and some biochemical characteristics of PCOS; i.e. LH to FSH ratios and FAI. These data further emphasize the significance of augmented androgen levels in peripheral blood or inside the ovary - as a consequence of increased theca cell stimulation by high LH levels (Erickson and Yen, 1984) - in the etiology of polycystic changes in the ovary. A higher mean number of follicles was observed in most cases with higher values of F-G score and BMI, but this association did not reach the level of statistical significance. Accordingly, the question what should be the threshold follicle number for the ovary to be considered as polycystic was not adressed.

The maximal follicle size observed in this study was 9.4 mm. This is in accordance with information provided by ultrasound literature, in which follicle sizes up to 10 mm in diameter were reported in women with polycystic ovaries (Swanson et al. 1981; Hann et al. 1984; Parisi et al.1984; Orsini et al. 1985; Adams et al. 1986; Tabbakh et al. 1986; Yeh et al. 1987). Mean follicle size was not related to mean follicle number indicating that the extent to which individual follicles grow is not related to the number of maturing or atretic follicles present.

Selection of a dominant follicle, as observed by transvaginal sonography, takes

place below 9.9 mm in diameter (Pache et al. 1990). Our observations in three subsequent sonography examinations performed within a 9 to 12 days period substantiate a non-progression of follicle growth. One may reasonably assume that this arrest of folliculogenesis, leading to chronic anovulation in polycystic ovaries, corresponds to disrupted selection. Moreover, a mean follicle size remaining remarkably constant in sequential examinations may also suggest the process of atresia is slowed down in these patients. In addition, very similar results in all ultrasound parameters observed between all three sonography examinations indicate that no benefit would be gained from sequential examinations in common clinical practice.

Our findings demonstrate the association between follicle number and ovarian volume and stroma echogenicity, and point to a correlation between follicle number and some biochemical features of PCOS in women with abnormal cycles. These data, along with a careful perusal of literature, emphasize the need to reconsider and further define ultrasonographic criteria for the diagnosis of polycystic ovaries in women with cycle abnormalities.

Summary

Transvaginal ultrasound examinations were performed in 37 oligo or amenorreic women to describe ovarian changes and potential correlations with clinical and some biochemical features of polycystic ovarian syndrome (PCOS). Findings were compared between the right and left ovary and between each of three subsequent examinations of the same ovary. In any one ovary, no significant difference was found between mean ovarian volume, stroma echogenicity, mean follicle number and size. Correlation between ovarian volume and follicle number was strong in all examinations. Follicle number in both ovaries correlated significantly with ovarian stroma echogenicity and some biochemical markers of PCOS. These observations validate the usefulness of transvaginal sonography in demonstrating numerous correlations in menstrual cycle disturbances. The strong necessity to reevaluate sonographic criteria of PCOS is highlighted.

6.4 How to discriminate between normal and polycystic ovaries. A transvaginal ultrasound study

Introduction

Since the sonographic diagnosis of polycystic ovaries may be used as one of the criteria for the diagnosis of polycystic ovary syndrome (PCOS), this method has gained wide acceptance in clinical practice. Until now, most reported findings were based on transabdominal sonography. However, studies concerning follicle number, ovarian size and stroma echogenicity have shown variable results (Fauser 1991a). It was recently stated that using the transabdominal approach ovarian architecture could not be adequately determined in as much as 42% of cases (Hull 1989). Underlying causes

were obesity, limited resolution of low frequency transducers, a full bladder distorting pelvic anatomy (Timor-Tritsch et al. 1988), or bowel loops adjacent to the ovary. High-frequency transvaginal ultrasound has the advantage of improved resolution and visualization (Yee et al. 1987; Coleman et al. 1988), and of better patient acceptance (Goldstein 1990). The use of transvaginal sonography to assess ovarian appearance in regular and irregular cycles, has recently been described (Pache et al. 1990; Pache et al. 1991a).

The objective of the present study was to determine cut-off levels for ovarian follicle size and number, ovarian echogenicity and volume, in women with PCOS by means of transvaginal sonography.

Subjects and methods

This study was approved by the local Ethics Review Committee. Twenty-nine regularly cycling women (median 28 days; range 25 to 31 days) with no previous history of infertility consented to participate in the study. They were recruited in 1989 and 1990 through advertisement. None of these women had received hormonal treatment for at least 4 months before the study. Their age ranged between 21 and 40 years (median 32 years), body mass index varied between 19.0 and 25.0 kg/m^2 (median 21.4 kg/m^2), and there was no hirsutism (Ferriman & Gallwey [1961] scores ranging between 1 to 6 [median 3]). Mean (±SD) serum levels of luteinizing hormone (LH), and testosterone (T), were 4.8 ± 1.7 IU/L, and 1.4 ± 0.6 nmol/L, respectively.

During the same period, 96 female patients consecutively attended the out-patient Clinic, because of infertility and cycle disturbances. Of these, 52 patients were diagnosed as presenting with PCOS, on the basis of LH and/or T values above 2 SD of the mean from the controls. Their age ranged between 18 and 41 years (median 27 years). Body mass index varied between 19.7 and 42.2 kg/m^2 (median 24.7 kg/m^2), and was > 25 kg/m^2 in 23 patients. The Ferriman and Gallwey score ranged between 1 and 29 (median 11), and was ≥ 8 in 38 patients. None of the subjects had been receiving treatment for at least 4 months before entering the study. 26 out of 52 patients were oligomenorrheic, and their cycle length ranged between 35 and 99 days (median 84 days). In the remaining 26 patients, amenorrhea had been present for more than 6 months. Mean serum concentrations of LH, and T, were 11.0 ± 5.1 IU/L, and 2.9 ± 1.2 nmol/l, respectively.

A 5 MHz transvaginal transducer (Orion; Philips Medical System, Eindhoven, The Netherlands) was used to perform a single ultrasound examination of the ovaries between cycle day 4 and 11 (median 8) in both regularly cycling women, and oligomenorrheic patients. Random ultrasound examination took place in the amenorrheic patients. All scans were carried out by the same observer (T.D.P.). Using the scanning method described below, the accuracy and reproducibility of follicle size measurements have been described previously (Pache et al. 1990). The number of follicles was established by scanning each ovary from the inner to the outer margin in longitudinal cross-sections. Depending on the longitudinal diameter of the follicle (≤

6.0, or > 6.0 mm, respectively), its size was determined from the mean of two dimensions (longitudinal and antero-posterior), or from the mean of three dimensions (longitudinal, antero-posterior, and transverse). Echogenicity was scored as normal (=1), moderately increased (=2), or markedly increased (=3) (8). From the longitudinal (A), antero-posterior (B), and transverse (C) dimension of the ovary, its volume was calculated according to the formula ½ x (A x B x C) (Sample et al. 1977).

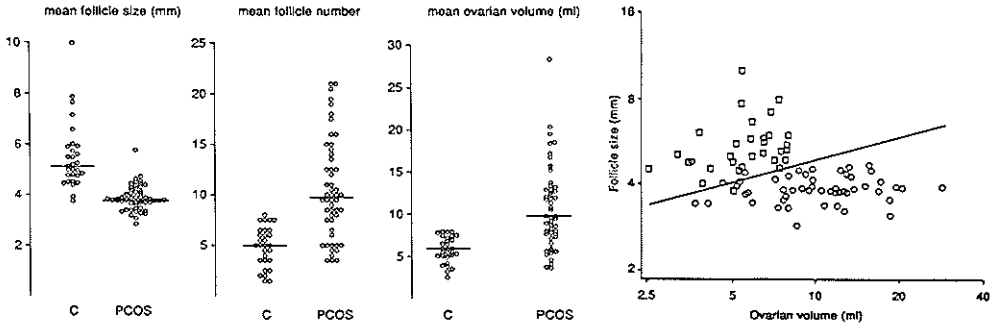
Blood samples were obtained the same day the ultrasound examination took place. Serum was assayed for LH and T. Commercially available radio-immunometric kits were purchased from Medgenix (Fleurus, Belgium) to determine levels of immunoreactive LH as previously described (Fauser et al. 1991b). Radioimmunoassays were performed to measure T as described by Verjans et al. (1973). Intra and interassay coefficients of variation were less than 5 and 15% for LH, and less than 3 and 5% for T, respectively.

The Mann-Whitney's test was used to perform univariate comparisons between groups. Multivariate logistic discriminant analysis (Anderson 1974) was applied to differentiate between controls and PCOS patients on the basis of ultrasound data. The a priori probability of polycystic ovaries in this analysis was set at 50%. The Chi-square test (2 df) was used to assess differences in ovarian stroma echogenicity between both groups. P values are two-sided, with 0.05 taken as the limit of statistical significance.

Results

No significant difference in median follicle number, follicle size, ovarian volume and in ovarian stroma estimates was observed between the 26 oligomenorrheic and 26 amenorrheic patients; they were therefore analyzed together. Also, no significant difference was observed between the right and the left ovary for all ultrasound parameters. Therefore, averaged values from both ovaries were used for further analysis. Distribution of the mean follicle number, size, and ovarian volume in controls and PCOS is displayed in Figure 1. No more than 11 follicles could be observed in any one ovary of the regularly cycling controls. These control ovaries were never found to exhibit a volume above 8.0 ml. Median values of mean follicle size and number were 3.8 mm and 9.8 in PCOS against 5.1 mm and 5.0 in controls ($P<0.001$). Median value for the mean ovarian volume was 5.9 ml in controls, and 9.8 ml in PCOS ($P<0.001$). In controls, ovarian stroma echogenicity was normal in 90%, and only moderately increased in 10%, whereas in PCOS echogenicity of the ovarian stroma was markedly increased in 54%, moderately increased in 40%, and normal in only 6%. This difference was statistically significant ($P<0.001$). When patients were classified according to the presence of moderately or markedly increased echogenicity, the sensitivity and specificity of ovarian stroma echogenicity in the diagnosis of polycystic ovaries was 94%, and 90%, respectively.

Considerable overlap between control women and PCOS patients existed for follicle number and size, and ovarian volume (Figure 1). None of these parameters



Figures 1, 2. (1) Distribution of mean follicular size, mean number of follicles, and mean ovarian volume in 29 women with regular menstrual cycles (C) and in 52 patients with PCOS. Each circle represents averaged data from both ovaries in one individual. Examination with transvaginal sonography was performed between cycle days 4 and 11 in control subjects. The horizontal bars indicate median values for each group. (2) Follicular size and ovarian volume in 29 women with regular menstrual cycles (□) and 52 patients with PCOS (○). Each data point refers to mean size of all follicles in both ovaries and to mean volume of both ovaries in one individual. Diagonal line [log(size) = 0.42 + 0.27 log(volume)] corresponds to values at which the likelihood of polycystic ovaries equals 50%.

could therefore be used separately as a single reference to discriminate between normal and polycystic ovaries. By comparing PCOS patients with control women using multivariate analysis, it appeared possible to classify most patients in the appropriate group. The greatest power in discriminating between PCOS patients and control women could be obtained by the combined measurement of follicle size and ovarian volume (Figure 2), whereas no significant (p=0.17) additional predictive value was present for follicle number. Classifying patients according to whether the data-points were situated below or above the line corresponding to a 50% probability of having polycystic ovaries (Figure 2), a sensitivity of 92% (48/52), and a specificity of 97% (28/29) was obtained.

Discussion

In the present investigation, transvaginal sonography was used to evaluate follicle number and size, ovarian volume and stromal aspect in regularly cycling women, and in patients with PCOS. Both infertility and cycle disturbances were present in all PCOS patients studied. These characteristics were therefore overrepresented when compared with data from other clinical series (Goldzieher & Axelrod 1963; Franks 1989; Conway et al. 1989). The incidence of hirsutism (73%) and obesity (44%) was rather similar.

Until now, most studies were carried out using the transabdominal route to diagnose polycystic ovaries. The number of follicles necessary to establish the diagnosis of polycystic ovaries has been reported to vary between "above 5" (Yeh et al. 1987), "more than 10" (Adams et al. 1985), and "at least 15" (Fox et al. 1991). In the present study, not more than 11 follicles could be detected in normal ovaries. On the other hand, a considerable number of ovaries from PCOS patients contained less than 11 follicles. It may therefore be considered that, using the present scanning technique, the specificity of a threshold value of $\geq$ 12 follicles per ovary is high,

whereas the sensitivity of this cut-off level is too low to rule out the diagnosis of polycystic ovaries in case a smaller number of follicles is observed. In a recent review of the morphology of polycystic ovaries, the presence of twice the number of all types of antral follicles generally under 4 mm, and twice the ovarian volume compared to control ovaries, was described (Hughesdon 1982). Our data are in close keeping with these histological values. The presence of only small follicles in our PCOS patients is in agreement with absent selection of the dominant follicle as described in polycystic ovaries (Goldzieher 1981).

Transabdominal sonography in PCOS patients revealed enlarged ovaries in 2.5% (Swanson et al. 1981) to 70% (Tabbakh et al. 1986) of the cases, and mean ovarian volumes between 12 and 14 ml (Hann et al. 1984; Adams et al. 1985; Orsini et al. 1985; Polson et al. 1988). Ovaries in PCOS patients appeared to be somewhat smaller when studied transvaginally. The wide overlap in size between normal and polycystic ovaries in the present study suggests that the discriminative power of ovarian volume parameter alone is also insufficient for the diagnosis of polycystic ovaries.

The role of the increased echogenicity of ovarian stroma as a marker of polycystic ovaries has been emphasized before (Adams et al. 1985; Hull 1989; Fox et al. 1991). It was found to be the most sensitive and specific sonographic sign of polycystic ovaries (Pache et al. 1991a; Ardaens et al. 1991). This is supported by the data from the present study. However, appraising ovarian stroma echogenicity is purely subjective, which could represent a drawback in clinical practice (Hull 1989).

The aim of the present investigation was to provide sonographic criteria for the diagnosis of polycystic ovaries. However, it has become evident that there is more biological variability than strict criteria would allow, and no cut-off level could be obtained for any of the quantitative parameters, which would have provided both a satisfactory sensitivity and specificity. As a consequence, combinations of parameters were explored, resulting in the most sensitive and specific combination being presented by follicle size and ovarian volume. It can be concluded that transvaginal sonography reliably offers the opportunity to diagnose polycystic ovaries. This may be helpful for a better understanding of mechanisms underlying PCOS and may allow detection of patients at risk (Raj et al. 1978) during induction of ovulation.

Summary

Cut-off levels for ovarian follicle size and number, ovarian echogenicity and volume, were estimated in 29 regularly cycling women (controls) and 52 patients with the polycystic ovary syndrome (PCOS). Median values for mean follicle number and size, and ovarian volume, were 5.0, 5.1 mm, and 5.9 ml in controls, and 9.8, 3.8 mm, and 9.8 ml in PCOS patients. Ovarian stroma echogenicity was normal in 90%, and moderately increased in 10 % of controls, whereas in PCOS echogenicity displayed a marked increase in 54%, a moderate increase in 40%, and was normal in 6%. Sensitivity and specificity of the presence of augmented stroma echogenicity in the diagnosis of polycystic ovaries was 94%, and 90%, respectively. The subjective nature

of stroma echogenicity appraisal, and the wide overlap in follicle number and size and ovarian volume between both groups may render the use of one single sonographic criterion to diagnose polycystic ovaries as insufficient. The greatest power of discrimination between normal and polycystic ovaries was obtained by combined measurement of follicle size and ovarian volume, with a sensitivity of 92% and a specificity of 97%. Transvaginal sonography reliably offers the opportunity to diagnose polycystic ovaries by using this combination of ultrasound parameters.

Chapter 7

Peripheral and intra-ovarian disturbances in women with polycystic ovaries

7.1 Introductory remarks

Estimates of immunoreactive serum concentrations of LH (I-LH) and FSH (I-FSH) are considered to be important diagnostic criteria for the diagnosis of PCOS. As early as 1958 periods of increased urinary output of LH-like bioactivity were described in a longitudinal study in patients suffering from the Stein-Leventhal syndrome (McArthur et al. 1958; Taymor & Barnard 1962). The introduction of specific radioimmunoassays (RIA) for LH subsequently enabled to disclose the presence of significantly higher I-LH serum concentrations in these patients (Yen et al. 1970). This finding was confirmed in numerous studies (Gambrell et al. 1973; DeVane et al. 1975; Baird et al. 1975; Rebar et al. 1976; Rebar 1984), although it was also noted that patterns of I-LH secretion varied from patient to patient, and that a proportion of these patients exhibited I-LH concentration within the normal range. Gonadotropins are secreted in a pulsatile fashion. However, normalcy of the results may not be due single hormone estimate at an inappropriate time of the cycle - or of the day - since even when frequent sampling is performed, I-LH pulse patterns appear to be normal in 20% of the cases (Franks & Polson 1989). Gonadotropins are also secreted as a heterogenenous group of isohormones with different half-lives and biological activities. In a number of cases, I-LH levels may not adequately quantify the biologically active hormone (Fauser & de Jong, 1993b), and more basic LH forms could underlay high LH bioactivity in PCOS women (Ding & Huhtaniemi 1992). Since animal studies suggest that gonadal steroids may influence both quantity and biopotency of secreted gonadotropins, it may be suspected that elevated androgen levels - as commonly found in PCOS women - would affect LH bioactivity. But bio- to immuno LH ratios have

been described to decrease or increase following T administration in man, and this issue remains controversial (Tsatsoulis et al. 1991).

Serum levels of I-FSH have been reported to be low (Yen et al. 1970) to low normal (Baird et al. 1975; Rebar et al. 1976) in PCOS women. Reports on bioactive FSH are not presently available in this type of patients. An elevated I-LH/I-FSH ratio - most often reported > 3 as measured by RIA methods - has been proposed as a diagnostic criterion for PCOS (Lobo et al. 1981; Lobo et al. 1984). As the use of immunoradiometric assay (IRMA) is expanding, it could be interesting to reevaluate to what extent old reference values for LH and LH/FSH ratios may still be trusted. Besides, FSH secretion and action may well be a key factor in the pathogenesis of PCOS. Under normal circumstances, FSH action at the ovarian level is cardinal to stimulate mitogenic activity of granulosa cells and induce aromatase enzyme activity, and both these elements appear crucial for ongoing follicular growth up to ovulation. In polycystic ovaries, follicular growth is disturbed, and selection commonly does not take place. However, exogenous gonadotropin administration generally leads to ovulation in PCOS women, thereby indicating that FSH action may be disturbed in these patients. This could be due either to changes in FSH bioactivity, or to dysfunction at the follicular level. In this regard, there is in vitro evidence that FSH dysfunction may exist at the follicular level in PCOS: it has been shown that addition of FSH to non-growing cultured granulosa cells obtained from polycystic ovaries led to further growth and increase in E2 production (Erickson et al. 1979).

Given the above, studies presented in this chapter 7 will address issues regarding LH and FSH immuno- and bioactivity in serum of PCOS women (chapter 7.2 and 7.3). The impact of gonadotropin action on granulosa cells will be estimated by follicular fluid estimates (chapter 7.5 and 7.6).

Knowing the limitations of the method, transvaginal ultrasound examination (TVS) of the pelvis has become a substitute to histology to assess the ovarian structure, and can be used to diagnose polycystic ovaries (see chapter 6). As mentioned above and in previous chapters from the present thesis, LH and T values are very often used as diagnosis criterion of PCOS. In this context, to what extent polycystic changes estimated by TVS play an additional or a complementary role in the panel of tools for PCOS diagnosis should be evaluated. The study presented in chapter 7.4 will address this issue, and further underscore the role that TVS examination of the ovaries may have in correlating ovarian structure with function.

7.2 Serum bioactive and immunoreactive luteinizing hormone and follicle-stimulating hormone levels in women with cycle abnormalities, with or without polycystic ovarian disease

Introduction

Diagnosis and classification of polycystic ovarian disease (PCOD) remains controversial since this syndrome involves a heterogenous group of patients and clear,

well validated diagnostic criteria are limited (Fauser 1991). Signs and symptoms are considered to be the main clinical standards for PCOD diagnosis. Next to high serum androgen levels in the majority of patients suffering from PCOD, the significance of inappropriate gonadotropin secretion (i.e. selectively increased luteinizing hormone (LH) levels), has been emphasized (Yen et al. 1970b; Rebar 1984). Although some PCOD patients appeared to have normal LH concentrations (Rebar 1984), augmented LH levels (and LH/follicle-stimulating hormone [FSH] ratios (Lobo et al. 1981; Lobo et al. 1984b) - as estimated by radioimmunoassay (RIA) - have been considered the crucial biochemical criterion for the diagnosis of PCOD. The recent availability of more selective immunological techniques for the detection of gonadotropins using monoclonal antibodies (immunoradiometric assay [IRMA]), together with the use of in vitro bioassays - by which the functional activity of circulating gonadotropins can be estimated - made hormone estimates obtained by classical RIAs in various clinical and pharmacological conditions questionable (Fauser et al. 1989; Jaakolla et al. 1990).

Altered urinary excretion of LH bioactivity in Stein-Leventhal syndrome patients has been described over 3 decades ago (McArthur et al. 1958). In PCOD patients elevated bioactive LH levels and increased bioactive to immunoreactive LH ratios have recently been reported (Lobo et al. 1983; Lobo et al. 1984b). Information obtained in these studies should be revised since discrepancies between various immunological techniques were recently established (Jaakolla et al. 1990), and LH levels - as measured by RIA - could have been overestimated because of considerable cross-reactivity of free circulating α-subunits in these assays (Meldrum et al. 1984b).

No data are presently available on gonadotropin levels estimated by IRMA and bioactive FSH levels in this type of patients. Bioactivity of circulating FSH seems to be of special interest since the previously described aromatase defect in ovaries of PCOD patients (Short and London 1961; Axelrod and Goldzieher 1962) together with observed normal FSH-induced aromatase activity of cultured granulosa cells obtained from PCOD patients (Erickson et al. 1979; Mason et al. 1990), suggest diminished FSH bioactivity either in the circulation or within ovarian follicles. The major objective of the present study was to estimate circulating gonadotropin concentrations using various immunological techniques and in vitro bioassays in patients with cycle abnormalities without and with PCOD according to strict clinical and biochemical criteria.

Materials and methods

Subjects and study protocol

Eight regularly cycling, young, healthy women (controls) and 35 women attending our Infertility Clinic presenting with oligomenorrhea (n=21; mean cycle length 53±4 days) or amenorrhea (n=14; no vaginal bleeding for at least 6 months) and infertility (3.5±2.6 yrs) were enrolled in the study (see Table 1). Underweight women (body mass index (BMI) < 19 kg/m^2) (Thomas et al. 1976) were excluded. In the healthy volunteers no endocrine disease was present, and no medication was taken for

TABLE 1. Clinical and steroid hormone observations (mean ± SD) in controls and women with cycle abnormalities, with or without PCOD

	Control (n = 8)		Oligo/amenorrhea (n = 35)	
	EFP	MFP	PCOD (n = 11)	Non-PCOD (n = 24)
Age (yr)	27.6 ± 4.1		25.8 ± 3.1	28.6 ± 4.9
BMI (kg/m²)	21.8 ± 1.6		29.9 ± 5.5	23.5 ± 4.7
F-G score	6.3 ± 1.0		14.5 ± 6.5	11.2 ± 1.5
Cycle length (days)	27.9 ± 1.8		—[a]	54 ± 3[b]
FAI (T × 100/SHBG)	1.6 ± 0.5	1.9 ± 0.7	11.2 ± 4.0	4.8 ± .5
A (nmol/L)	8.8 ± 3.1	9.7 ± 4.1	20.2 ± 8.7	12.3 ± 5.6
DHEAS (μmol/L)	—	—	9.7 ± 4.7	7.4 ± 3.5
E_1 (pmol/L)	272 ± 47	292 ± 31	489 ± 119	325 ± 113
E_2 (pmol/L)	96 ± 29	170 ± 29	236 ± 72	158 ± 62

EFP, EF phase (cycle days 3–4); MFP, MF phase (cycle days 7–8); F-G, Ferriman and Gallwey score (15).
[a] Not indicated (amenorrhea in eight women).
[b] Six women with amenorrhea were excluded.

at least 6 months.

The study was approved by the Ethics Review Committee of the Erasmus University, and informed consent was obtained from all participants. In the eight control women ovarian sonography (Pache et al. 1990) and daily blood withdrawal was performed (data not shown). Single gonadotropin and steroid hormone levels are reported here for the early follicular (EF; cycle day 3 or 4), and mid follicular (MF; cycle day 7 or 8, according to the occurrence of the LH peak) phase only, in agreement with comparison of LH levels by Kazer et al. (1987). Mid-luteal progesterone levels were above 30 nmol/L in all control women (data not shown). Ovarian transvaginal sonography and single bloodwithdrawal was performed shortly after menses (cycle day 3 or 4) in oligomenorrheic patients and at random in the patients with amenorrhea. Samples were collected within a 6 months period. Patients with cycle abnormalities were divided in PCOD and non-PCOD based on the following criteria: (1) Obesity; BMI ≥ 26. (2) Hirsutism; Ferriman and Gallwey (F-G) score ≥ 8 (1961). (3) High androgen levels; Dehydroepiandrosterone sulphate (DHEAS) ≥ 10 μmol/L, and/or testosterone (T) x 100 / sex-hormone binding globulin (SHBG) (free androgen index [FAI]) ≥ 5 (Eden et al. 1989). (4) Ovaries bearing multiple cysts as observed by transvaginal sonography; arbitrarily chosen as number of follicles per ovary ≥ 12 (the maximum number of follicles previously observed in normal ovaries was 11 (Pache et al. 1990), in combination with increased ovarian stroma echogenicity. It was recently shown that the mean follicle number in ovaries of women with menstrual dysfunction observed by sonography is 10, and the follicle number correlates with ovarian stroma echogenicity, FAI, and LH/FSH ratios (Pache et al. 1991). Patients scoring positive in at least 3 out of the 4 above mentioned criteria were diagnosed as having PCOD (see also Table 2). All PCOD and non PCOD patients had thyroid-stimulating hormone and prolactin (except one in the non-PCOD group) levels within normal limits (data not shown).

Hormone assays

Immunoreactive LH was determined using a commercially available RIA kit (Amersham, UK). FSH RIA was performed as described previously (Klijn et al. 1980),

TABLE 2. Clinical and biochemical features of PCOD patients

Patient no.	Age (yr)	BMI (kg/m^2)	F-G score	Cycle length (days)	Ultrasound[a]	FAI	DHEAS (μmol/L)
1	29	20.4	12	—[b]	+	11.7	15.8
2	26	26.2	4	—	+	11.0	7.1
3	26	37.7	9	—	+	13.8	13.0
4	23	31.2	8	—	−	6.8	4.7
5	30	23.5	16	67	+	7.2	5.5
6	28	38.6	27	—	−	20.5	10.9
7	27	26.8	17	—	+	12.9	15.6
8	24	26.0	12	35	−	8.8	14.7
9	18	32.4	22	—	+	7.3	1.8
10	27	33.9	12	—	−	9.6	10.0
11	26	32.4	21	42	+	14.0	7.8

F-G score, Ferriman and Gallwey score (18).
[a] Appearance of polycystic ovaries.
[b] Amenorrhea.

using an antiserum purchased from UCB (Brussels, Belgium). Intra- and interassay coefficients of variation were less than 5 and 10% for LH, and less than 8 and 11% for FSH, respectively. IRMA LH and FSH kits were from Medgenix (Fleurus, Belgium) as described earlier (Fauser et al. 1990). Data are expressed in terms of MRC 68/40 reference preparation for LH and MRC 78/549 for FSH. Intra- and inter-assay coefficients of variation were less than 5 and 15% for LH, and less than 3 and 8% for FSH, respectively. Cross reactivity with α-subunit was 0.4% for LH, and 0.06% for the FSH IRMA.

Bioactive LH and FSH levels in serum samples were measured using the in vitro mouse Leydig cell (van Damme et al 1974) and rat granulosa cell (Dahl et al. 1989) bioassays. The reference preparations (human pituitary gonadotropin LER-907; FSH biopotency 20 IU/mg, LH biopotency 60 IU/mg using the second IRP of human menopausal gonadotropin standards) were obtained from the National Hormone and Pituitary Distribution Program, NIDDK. As was shown previously (Fauser et al. 1990) the bioassay standard curve was parallel for the IRMA standard (78/549) as compared to LER-907. Each serum sample was assayed in triplicate at three dose levels. All bioassay samples were run in the same assay, with intra-assay variations of 12% for LH and 10% for FSH.

The α-subunit assay kit was from UCB (Brussels, Belgium) as described previously (Kwekkeboom et al. 1990). Intra- and interassay coefficients of variation were less than 6 and 11%, and cross reactivity of the α-subunit assay with LH and FSH was 3.9% and 20.0%, respectively. All steroids were measured by RIA. Estrone (E1) and T measurements were described previously (van Landeghem 1981). SHBG, estradiol (E2), androstenedione (A), and DHEAS kits were from Diagnostic Products Corp. (Los Angeles, CA). Intra- and interassay coefficients of variation were less than 4 and 5% for SHBG, less than 11 and 14% for E1, less than 5 and 7% for E2, less than 8 and 11% for A, less than 3 and 5% for T, and less than 4 and 6% for DHEAS, respectively.

Data analysis

Data are presented as mean ± SD if they are normally distributed, and as median and range if distributed otherwise. Comparisons of means or medians between groups were done using Mann-Withney's test and within groups with the Wilcoxon's test. To eliminate the effect of outlying observations correlation coefficients given are Spearman's (r_s). P values given are two-sided, with 0.05 taken as limit for statistical significance.

Results

Controls

Clinical information and serum concentrations of steroid hormones, immunoreactive (RIA, and IRMA) and bioactive LH and FSH, and α-subunits in the EF and MF phase of 8 normally cycling women have been summarized in Tables 1 and 3. Individual BIO-LH levels were all below 9.1, except one. Except for a 1.9-fold increase in mean E2 levels in the MF as compared to the EF phase, steroid levels did not change significantly.

Patients with cycle abnormalities, not diagnosed as PCOD

Steroid hormone, LH, FSH, and α-subunit serum levels and clinical information of 24 patients suffering from oligomenorrhea or amenorrhea not diagnosed as PCOD have been indicated in Tables 1 and 3. Except for augmented FAI ($P<0.03$), no significant changes in steroid levels could be observed as compared to normal controls. In these 24 patients, individual levels were above the normal range in 8 patients for FAI, in 4 subjects for A, in 4 subjects for DHEAS, and in 6 subjects for E1. As compared to the EF phase of control cycles, mean IRMA-LH and RIA-LH levels in non-PCOD patients were moderately elevated (2.2-2.5-fold, respectively. Both comparisons, $P\leq0.03$), whereas the median BIO-LH level remained unaltered. Therefore BIO/IRMA-LH ratios for non-PCOD patients as compared to the EF phase of control

TABLE 3. Immunoreactive [IRMA (I), and RIA (R)] and bioactive (B) serum LH and FSH levels, LH/FSH ratios, and α-subunit concentrations in controls and women with oligo/amenorrhea with or without PCOD

	Control (n = 8)		Oligo/amenorrhea (n = 35)	
	EFP	MFP	PCOD (n = 11)	Non-PCOD (n = 24)
I-LH (IU/L)	3.6 ± 1.4	5.1 ± 2.3	9.9 ± 3.9	7.9 ± 5.2
R-LH (IU/L)	5.0 ± 1.3	6.6 ± 1.2	17.8 ± 4.5	12.4 ± 8.0
B-LH (IU/L)	4.8 (1.0–9.1)	6.1 (1.3–33.0)	19.0 (2.2–33.0)	3.2 (1.0–33.0)
B/I-LH	1.3 (0.3–1.9)	1.5 (0.4–4.3)	1.9 (0.3–25.4)	0.4 (0.3–3.3)
I-FSH (IU/L)	5.8 ± 2.4	5.2 ± 1.4	5.8 ± 2.0	5.9 ± 2.8
R-FSH (IU/L)	4.0 ± 1.3	3.5 ± 1.1	4.0 ± 0.7	4.3 ± 1.1
B-FSH (IU/L)	34.4 ± 14.3	27.2 ± 8.8	22.6 ± 11.4	19.5 ± 10.7
B/I-FSH	6.1 (2.6–13.1)	5.7 (2.8–7.5)	3.7 (1.4–8.8)	2.6 (1.2–19.6)
I-LH/FSH	0.5 (0.4–1.5)	0.8 (0.5–2.1)	1.8 (0.3–4.1)	1.2 (0.2–3.1)
R-LH/FSH	1.3 ± 0.4	2.0 ± 0.5	4.5 ± 1.3	2.9 ± 1.6
B-LH/FSH	0.2 (0.02–0.5)	0.2 (0.04–1.7)	1.0 (0.1–3.5)	0.2 (0.1–2.5)
α-Subunit (μg/L)	0.1 (0.1–0.6)	0.2 (0.1–0.7)	0.9 (0.2–1.9)	0.5 (0.1–1.3)

EFP, EF phase (cycle days 3–4); MFP, MF phase (cycle days 7–8).

PCOD patients mean IRMA-LH and RIA-LH levels are in the same order of magnitude as compared to levels in women with cycle abnormalities not diagnosed as PCOD. However, BIO-LH levels in PCOD patients were elevated as compared to controls, and distinctly augmented (5.9-fold increment) as compared to non-PCOD patients. Therefore a major discrepancy in BIO/IRMA-LH ratios was disclosed comparing these two patient groups.

Although RIA-LH levels were consistently higher than IRMA-LH concentrations, a good correlation was found between these immunological techniques in the group of women with cycle abnormalities (Fig. 1). Cross-reactivity of the conventional RIA with free circulating α-subunits might largely explain the observed differences between RIA and IRMA, since α-subunit levels were elevated in women with cycle abnormalities. In addition, LH isohormones with limited biological activity

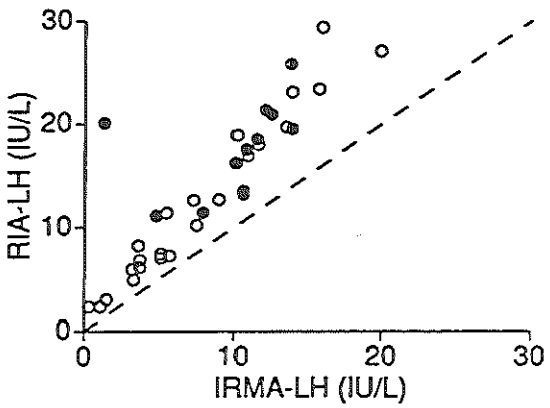


FIG. 1. Correlations ($r_s = 0.88$; $P < 0.001$) between IRMA- and RIA-LH levels in 35 women with menstrual cycle disturbances (●, PCOD patients; ○ non-PCOD patients). The *line* denotes the line of identity.

may also be recognized in the RIA. The correlation between RIA-LH and IRMA-LH, however, does not suggest that LH isohormones with high biological activity are recognized more selectively in the IRMA assay. Elevated immunoreactive LH/FSH ratios in PCOD patients as observed in the present study is in agreement with previous literature (Lobo et al. 1981; Lobo et al. 1984b). LH/FSH ratios in PCOD patients were higher when estimated by RIA (median 4.5), as compared to IRMA (mean 1.8). The relative increase of LH/FSH ratios in PCOD patients as compared to the EF phase of control cycles is, however, similar using RIA (3.5-fold) or IRMA (3.6-fold). Immunoreactive LH/FSH ratios were also elevated in non-PCOD patients as compared to the EF phase (2.2-2.4 fold; P≤0.04). The increase of LH/FSH ratios in PCOD patients as compared to non-PCOD subjects did reach statistical significance for RIA (P=0.008), and BIO (P=0.002), but not for IRMA. Obtained data suggest that LH/FSH

ratios - as a diagnostic criterion for the diagnosis of PCOD - should be reevaluated for IRMAs. Median BIO-LH level in PCOD patients was distinctly augmented as compared to the EF phase of control cycles (4.0-fold elevation) and non-PCOD patients (5.9-fold elevation). Since mean IRMA-LH and RIA-LH levels were only moderately elevated in non-PCOD patients median BIO/IRMA-LH ratio was clearly different when PCOD and non-PCOD patients were compared. This seems to be an important observation and it might be hypothesized that in PCOD patients LH isohormones are secreted which exhibit high biological activity, whereas immunological activity is relatively low. The presence of circulating molecules with LH-like bioactivity in PCOD patients could also explain the observed discrepancy. Indeed, the slope of the regression line after log-transformation between IRMA-LH as compared to BIO-LH (Fig. 2) is significantly greater for non-PCOD versus PCOD patients (0.82 versus 0.01, respectively; $P<0.05$). Further studies with larger numbers of patients are warranted to underline the present observations. Low (BIO- and RIA-) LH levels as observed in some PCOD patients are also in agreement with previous literature (Lobo et al. 1983; Rebar 1984), and may emphasize the heterogeneity of the group. In addition, single blood samples may underestimate LH concentrations due to the pulsatile nature of LH release. Only one patient in the non-PCOD group showed high BIO-LH levels (Fig. 2). Reevaluation of this patient showed that she could have been considered PCOD if sonographic criteria would have been choosen differently. Discrepancies with literature might occur by the present use of strict criteria for diagnosing PCOD. This further stresses the need for careful evaluation of diagnostic criteria because this may clearly affect observed differences between various patient groups studied.

Animal studies suggest that gonadal steroids - androgens as well as estrogens - may affect both biopotency and quantity of stored and released gonadotropic hormones (Peckham and Knobil 1976; Solana et al. 1980; Chappel et al. 1984). Observations in humans suggesting that gonadal steroids may affect the biopotency of released LH (but not FSH) have been reported by several authors (Strollo et al. 1981; Chang et al. 1984; Veldhuis and Dufau 1987; Tsatsoulis and al. 1988; Carani et al. 1989). In the present study in women with cycle abnormalities correlations could be observed between E2/SHBG ratios, FAI and BIO/IRMA-LH ratios. It seems therefore that also in humans alterations in steroid feedback may selectively affect - either directly at the pituitary gonadotrops or through changes in hypothalamic gonadotropin-releasing hormone secretion - biopotency of secreted LH. The observed correlation between DHEAS concentrations and BIO/IRMA-LH ratios is an extension of the previously described correlation between DHEAS and BIO-LH levels in control subjects and women with PCOD (Lobo et al. 1983). An explanation for this phenomenon remains highly speculative and warrants further studies. Although E1 levels do correlate with BIO-LH levels a correlation with BIO/IRMA-LH ratios could not be established.

α-Subunit concentrations are augmented in most clinical conditions where elevated LH levels are present. These conditions include the mid-cycle LH surge (Fauser et al., unpublished observations), spontaneous LH pulses, and the postmenopause (Kwekkeboom et al. 1990). Indeed, as shown now, PCOD patients

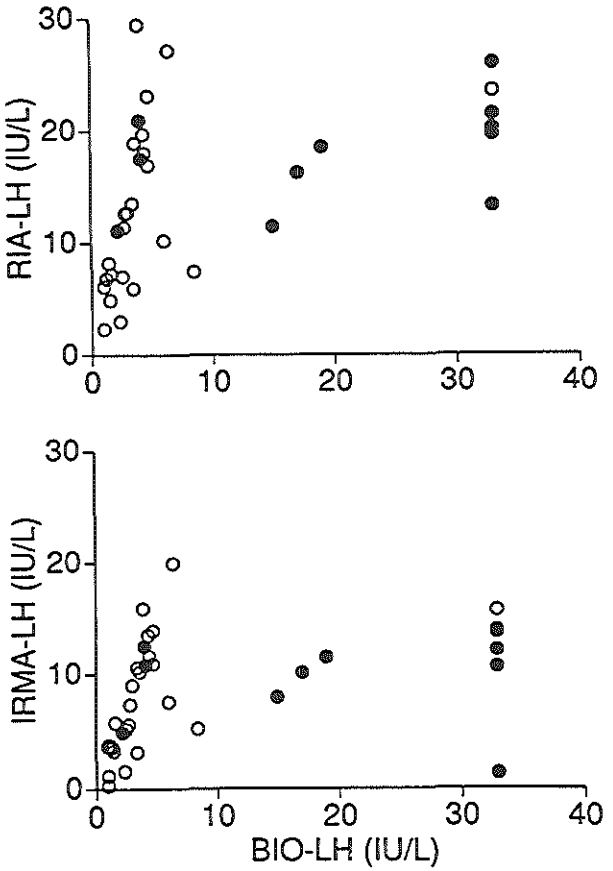


FIG. 2. Correlation between BIO- and RIA-LH (*upper panel*; $r_s = 0.72$; $P < 0.001$) and between BIO-LH and IRMA-LH serum levels (*lower panel*; $r_s = 0.64$; $P < 0.001$) in 35 women with menstrual cycle disturbances (●, PCOD patients; O, non-PCOD patients).

characterized by augmented LH secretion exhibit elevated α-subunit levels as well. Underlying regulatory mechanisms of synthesis and secretion of α-subunits and gonadotropins (Gharib et al. 1990), and the potential biological effects of elevated α-subunit levels remain speculative.

In the present study serum FSH levels were in the normal range in PCOD patients regardless whether they were estimated by RIA, IRMA or bioassay. Apparently alterations in gonadal steroid feedback does not affect FSH biopotency. It has previously been shown that ovaries of PCOD patients are incapable of converting androgens to estrogens (Short and London 1961; Axelrod and Goldzieher 1962), whereas aromatase activity of granulosa cells from PCOD patients can be induced in

vitro by FSH (Erickson et al. 1979; Mason et al. 1990). The presence of normal FSH bioactivity in serum of PCOD patients, as shown in the present study, points to disturbed intrafollicular FSH action in these patients.

In summary, data presented in this study suggest that highly bioactive LH isohormones, which may be regulated by altered steroid feedback activity, are secreted in most PCOD patients. PCOD should be considered a clinical model to study steroid regulation of LH biopotency. LH/FSH ratios are distinctly higher in PCOD patients if estimated by RIA as compared to IRMA. LH/FSH ratios should therefore be reevaluated as a diagnostic criterion for PCOD, and IRMA may not be useful to cycles tended to decrease (P=0.08).

PCOD patients

In PCOD patients mean FAI, and mean A, and E1 levels were significantly augmented as compared to non-PCOD patients and EF and MF phase concentrations in control cycles (for data see Table 1; all comparisons P<0.01). In the 11 women diagnosed as having PCOD, individual levels were above the normal range in all patients for FAI, in 8 subjects for A, in 6 subjects for DHEAS, and in 9 subjects for E1. Mean IRMA- and RIA-LH levels were elevated in PCOD patients as compared to controls (2.8- and 3.6-fold, respectively, for the EF phase. Both comparisons P≤0.001), but in the same order of magnitude (less than 1.4-fold difference; P=N.S.) as compared to non-PCOD patients (Table 3). Median BIO-LH level in PCOD patients was also augmented as compared to the level in non-PCOD patients (5.9-fold elevation; P<0.001), and in the EF phase of controls (4.0-fold elevation; P=0.002). Consequently, median BIO/IRMA-LH ratio in PCOD patients was 4.8-fold increased (P=0.004) as compared to non-PCOD patients. Mean IRMA-FSH, RIA-FSH, and BIO-FSH levels, and median BIO/IRMA-FSH ratios were not significantly different when various groups were compared.

In PCOD patients immunoreactive LH/FSH ratios were increased as compared to controls (1.8 vs. 0.5 for median IRMA-LH/FSH, and 4.5 vs. 1.3 for mean RIA-LH/FSH; both comparisons P≤0.01). Comparison between PCOD versus non-PCOD patients showed no significant difference in IRMA-LH/FSH ratios (1.5-fold difference; P=N.S.), but elevated RIA-LH/FSH ratios (1.6-fold; P=0.008), and BIO-LH/FSH ratios (5-fold; P=0.002) in PCOD. Median BIO-LH/FSH ratio in PCOD patients was elevated 5-fold as compared to controls (P=0.0003) and 5-fold as compared to non-PCOD patients (p=0.002).

Median α-subunit serum level in PCOD patients was augmented as compared to controls (9-fold elevation; P<0.001) and non-PCOD patients (1.8-fold elevation; P=0.05).

Correlations between various LH assays, and between LH and steroid levels in women with menstrual cycle disturbances

In the 35 women with menstrual cycle disturbances a strong correlation was observed between RIA-LH and IRMA-LH (r_s=0.88; P<0.001), although RIA-LH levels were consistently higher (Fig. 1). BIO-LH levels were correlated with RIA-LH (r_s=0.72; P<0.001) and IRMA-LH (r_s=0.64; P<0.001) (Fig. 2). BIO-LH levels were also

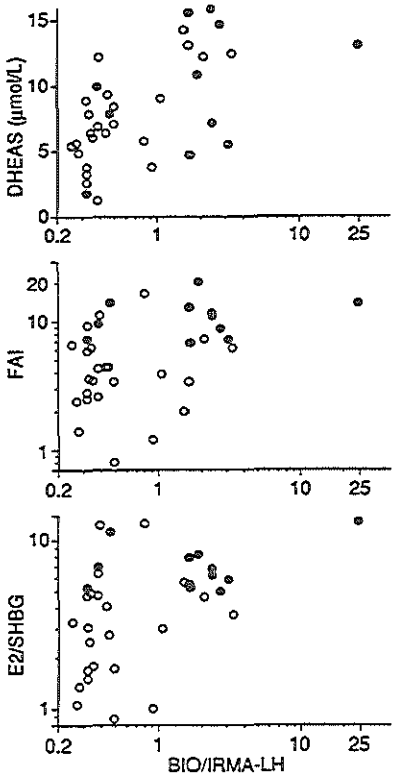


FIG. 3. Correlation between BIO/IRMA-LH ratios and DHEAS levels (*upper panel*; $r_s = 0.60$; $P < 0.001$), FAI (*middle panel*; $r_s = 0.39$; $P = 0.02$), and E_2/SHBG ratios (*lower panel*; $r_s = 0.47$; $P = 0.004$) in 35 women with menstrual cycle disturbances (●, PCOD patients, ○, non-PCOD patients).

correlated with FAI (r_s=0.54; P<0.001), T (r_s=0.73), E1 (r_s=0.67), and E2 concentrations (r_s=0.75), and E2/SHBG ratios (r_s=0.48; P=0.004). Furthermore, BIO/IRMA-LH ratios were correlated with BIO-LH levels (r_s=0.48), E2/SHBG ratios (r_s=0.47), FAI (r_s=0.39; P=0.02), and DHEAS concentrations (r_s=0.60) (Fig. 3).

Discussion

Results of this study in women with cycle abnormalities indicate elevated IRMA-LH, and RIA-LH serum concentrations as compared to normal controls. In identify PCOD patients. Normal serum FSH bioactivity - as estimated by the induction of aromatase activity in cultured rat granulosa cells - in these PCOD patients seems of major clinical relevance and stresses the possibility of disturbed FSH action within ovarian follicles in this condition.

Summary

Serum steroid, gonadotropin and α-subunit levels were assessed in 35 women with cycle abnormalities - 11 with and 24 without polycystic ovarian disease (PCOD) according to strict clinical and biochemical criteria - and 8 regularly cycling women in the early (cycle day 3 or 4) and mid (cycle day 7 or 8) follicular phase. Luteinizing hormone (LH) and follicle-stimulating hormone (FSH) levels were estimated using 2 immunological techniques (radioimmunoassay [RIA], and immunoradiometric assay [IRMA]) and in vitro bioassays (BIO), using mouse Leydig cells and rat granulosa cells, respectively. In PCOD patients mean α-subunits, free androgen index (FAI; testosterone x 100/sex hormone binding globulin [SHBG]), androstenedione, estrone, and estradiol (E2) were significantly elevated as compared to levels in the early follicular phase of control cycles and non-PCOD patients. In addition, in PCOD patients mean IRMA-LH and RIA-LH levels were distinctly increased (2.8-3.6 fold, respectively; both comparisons $P<0.001$) as compared to controls, but in the same order of magnitude (1.3-1.4 fold increments) as compared to non-PCOD patients. However, median BIO-LH level in PCOD patients was 5.9-fold higher as compared to non-PCOD patients and 4.0-fold higher as compared to BIO-LH in the early follicular phase of control women. Consequently, the median BIO/IRMA-LH ratio was 4.8-fold higher in PCOD patients as compared to non-PCOD patients. In women with cycle abnormalities individual BIO/IRMA-LH ratios did correlate with BIO-LH ($r_s=0.48$), FAI ($r_s=0.39$), free estrogens (E2/SHBG ratios) ($r_s=0.47$), and dehydroepiandrosterone sulphate (DHEAS) ($r_s=0.60$) concentrations. Mean IRMA-, RIA-, and BIO-FSH levels and BIO/IRMA-FSH ratios were not significantly different when various groups were compared. Although RIA- and IRMA-LH levels showed good correlation ($r_s=0.88$) RIA-LH levels were consistently higher, resulting in distinctly higher RIA-LH/FSH (mean 4.5) as compared to IRMA-LH/FSH ratios (median 1.8) in PCOD patients.

Results presented in this study indicate: 1) No differences in mean serum levels of IRMA-FSH, RIA-FSH, and BIO-FSH comparing PCOD, non-PCOD patients and controls. 2) LH/FSH ratio estimated by IRMA in PCOD patients is substantially lower than if measured by RIA. IRMA may not be useful to identify PCOD patients. 3) Free α-subunit levels in PCOD patients are elevated as compared to controls and non-PCOD patients. 4) BIO/IRMA-LH ratios in PCOD patients are significantly increased as compared to non-PCOD patients. 5) In women with cycle abnormalities BIO/IRMA-LH ratios are correlated with FAI, E2/SHBG ratios, and DHEAS levels.

7.3 The significance of serum luteinizing hormone measurements in women with cycle disturbances: discrepancies between immunoreactive and bioactive hormone estimates

Introduction

Elevated serum LH levels as observed in a proportion of women presenting with oligo/amenorrhea appear to have major clinical relevance, since the outcome of in vitro fertilization may be reduced (Howles et al. 1986), and the high rate of early pregnancy loss is associated with hypersecretion of LH (Homburg et al. 1988; Regan et al. 1990). Moreover, elevated LH levels are strongly associated with the polycystic ovary syndrome (PCOS) (Rebar 1984). Because of the controversy related to clinical and sonographic characteristics (Fauser 1991a), in clinical practice elevated LH levels - sometimes in combination with augmented testosterone (T) concentrations - are still considered the key diagnostic features for PCOS diagnosis by many authors. This diagnosis is clinically relevant since success rates of gonadotropin induction of ovulation are reported to be relatively poor in this group of patients (Homburg et al. 1988; Diamond & Wentz 1986).

Recent studies in PCOS patients revealed the presence of increased bioactive LH levels (Lobo et al. 1983; Lobo et al. 1984b; Antilla et al. 1991; Fauser et al. 1991b). Observations in humans under various circumstances, suggested LH bioactive to immunoreactive ratios - an index of LH biopotency (Fauser & Hsueh 1989) - to be affected by gonadal steroids (Dufau & Veldhuis 1987; Jaakolla et al. 1990). Interestingly, during androgen treatment of hypogonadal men a negative correlation between circulating T levels and the LH biopotency was observed (Tsatsoulis et al. 1990). Animal studies imply that gonadal steroids may act directly at the pituitary level to control both quantity and biopotency of stored and released gonadotropins. It is believed that oestrogens enhance GnRH stimulation of both LH polypeptide synthesis and glycosylation (Ramey et al. 1987), whereas T acts to selectively inhibit LH glycosylation (Krummen & Baldwin 1988). PCOS patients, often presenting with normal oestrogen and elevated T levels, may therefore be a suitable model for the study of androgen modulation of LH biopotency in peripheral blood.

This study was undertaken to extend previous observations in women with cycle abnormalities (Fauser et al. 1991b). The significance of endocrine diagnostic criteria - especially immunoreactive and bioactive LH estimates - for the characterization of PCOS patients is assessed.

Materials and methods

Subjects and study protocol

This study was approved by the Ethics Review Committee of the Erasmus University / Dijkzigt Hospital, and informed consent was obtained from all participating women. Ninety nine women (age; 27±5 [mean±SD] years) attending our

Infertility Clinic in 1990 presenting with infertility and oligomenorrhea (n = 58, cycle length > 35d) or amenorrhea (n = 41, no vaginal bleeding for at least 6 months) were asked to participate in this study. Thirty healthy normal weight regularly cycling women (age; 31±7 years, body mass index [BMI] 22.0±1.9 kg/m^2, cycle length 27.9±1.6 days) served as controls. Underweight women (BMI < 18 kg/m^2 [Thomas et al. 1976]) with presumably low serum gonadotropin levels were excluded. Ovarian transvaginal sonography (Pache et al. 1991a) and a single bloodwithdrawal was performed shortly after menses (between cycle day 4 and 8) in oligomenorrheic patients and controls and at random in patients with amenorrhea. All samples were collected between 9:00-11:00 h. within a 1 year period.

Prolactin levels were above normal in 6 women, and 4 patients exhibited elevated thyroid-stimulating hormone levels (data not shown).

Hormone assays

Immunoreactive LH was determined using a commercially available immunoradiometric assay (IRMA) kit (Medgenix, Fleurus, Belgium) as described previously (Fauser et al. 1990; Kwekkeboom et al. 1990a). Data are expressed in terms of the MRC 68/40 reference preparation, and intra- and interassay coefficients of variation were less than 5 and 15%. Sensitivity of the assay amounted to 0.5 IU/L, and cross-reactivities on a weight/weight basis were 0.5% for FSH, 0.4% for α subunit and <0.5% for the LH-ß subunit, as studied in postmenopausal women (Kwekkeboom et al. 1990a). Correlations of results of this assay with results of an in vitro Leydig cell LH bioassay were described earlier in women with cycle abormalities (Fauser et al. 1991b). It has also been shown that results of this assay correlate well with a "classical" radioimmunoassay in these women (Fauser et al. 1991b) and in male patients suffering from prostatic carcinoma treated with a gonadotropin-releasing hormone analog (Kwekkeboom et al. 1990b). Finally, IRMA-LH results have been described in oligospermic men (Fauser et al. 1990). Bioactive LH levels were estimated using the mouse Leydig cell assay (van Damme et al. 1974). The reference preparation (human pituitary gonadotropin LER-907; LH biopotency 60 IU/mg, using the second International Reference Preparation of human menopausal gonadotropin standards) was obtained from the National Hormone and Pituitary Distribution Program, NIDDK. As stated previously (Fauser et al. 1990; Fauser et al. 1991b), the bioassay standard curve was parallel for the IRMA standard as compared to LER-907. Each serum sample was assayed in duplicate at two dose levels. Intra-and interassay variations were 8 and 12 %, respectively.

All steroid hormones were measured by radioimmunoassay (RIA). Estrone (E1), T, sex-hormone binding globulin (SHBG), oestradiol (E2), androstenedione (AD), and dehydroepiandrosterone sulphate (DHEAS) measurements have been described earlier (Fauser et al. 1991b). Intra- and interassay coefficients of variation were less than 4 and 5% for SHBG, less than 11 and 14% for E1, less than 5 and 7% for E2, less than 8 and 11% for AD, less than 3 and 5% for T, and less than 4 and 6% for DHEAS, respectively.

Data analysis

Since controversy exists related to criteria for PCOS diagnosis, the following approach was decided upon. The group of women with cycle abnormalities was categorized according to: 1) clinical and endocrine criteria for PCOS diagnosis excluding LH (Table 1), as published previously (Fauser et al. 1991b). These criteria included: a) BMI, ≥ 26 kg/m^2, b) Ferriman & Gallwey score for hirsutism, ≥ 8, c) free androgen index (FAI; Tx100/SHBG) ≥ 5, and/or DHEAS ≥ 10 μmol/L, and d) polycystic appearance of ovaries by vaginal sonography (arbitrarily chosen as 12 or more follicles in any one ovary) (Pache et al. 1990; Pache et al. 1992). Since none of these criteria is believed to be obligatory, scoring had to be positive in at least 3 of these 4 criteria for PCOS diagnosis. 2) The presence or absence of elevated IRMA-LH levels (concentrations exceeding mean + 2SD [4.9 + 2x1.7 IU/L] of the control group (Fig. 1). In addition, the total study group was also divided into subgroups based on the presence or absence of elevated IRMA-LH and T levels. This, however, did not yield additional information as compared to groups classified on the basis of IRMA-LH levels alone (data not shown). 3) The presence of high or low BIO-LH concentrations (Table 3).

Data are presented as median and range, unless stated otherwise. Differences in quantitative data between groups were evaluated using Mann-Whitney's test. Fisher's exact test was used to compare percentages. Correlation coefficients given were calculated according to Pearson. This method was applied after logarithmic transformation of hormonal data to obtain approximately normal distributions. Multivariate analysis (logistic regression) (Cox 1970) was used to compare PCOS and non-PCOS patients with respect to IRMA-LH and BIO-LH simultaneously. P values given are two-sided, with 0.05 taken as the limit for statistical significance.

Table 1 Clinical and endocrine characteristics of 99 women presenting with infertility and oligo/amenorrhoea, separated into subgroups according to clinical (overweight, hirsutism, polycystic appearance of ovaries by sonography) and endocrine (elevated FAI levels) characteristics of PCOS

	PCOS* ($n = 35$)	non-PCOS ($n = 64$)	P value†
BMI (kg/m^2)	28·5 (18·1–48·7)	22·3 (18·3–42·2)	
Amenorrhoea	69%	27%	< 0·001
Polycystic ovaries‡	86%	28%	
IRMA-LH (IU/l)	8·5 (1·0–35·5)	5·4 (0·3–19·9)	0·07
BIO-LH (IU/l)	12·8 (1·1–64·4)	4·9 (1·0–67·1)	< 0·001
BIO/IRMA-LH	1·9 (0·3–25·8)	1·2 (0·2–5·3)	0·01
IRMA-FSH (IU/l)	4·2 (1·7–9·3)	4·6 (0·4–10·0)	0·4
IRMA-LH/FSH	1·6 (0·3–10·1)	1·2 (0·2–7·3)	0·06
T (nmol/l)	2·6 (1·6–7·6)	1·9 (0·6–3·9)	< 0·001
FAI	6·8 (2·1–20·5)	3·2 (0·4–16·5)	
AD (nmol/l)	14·8 (8·1–37·0)	11·1 (2·6–33·0)	< 0·001
DHEAS (μmol/l)	7·8 (1·8–15·8)	6·2 (0·3–15·6)	
E1 (pmol/l)	384 (193–713)	317 (137–777)	0·02
E2 (pmol/l)	208 (79–426)	174 (64–783)	0·1

* For criteria see materials and methods section.
† Features dependent on inclusion criteria were not statistically evaluated.
‡ Twelve or more follicles in one ovary, as determined by vaginal sonography.

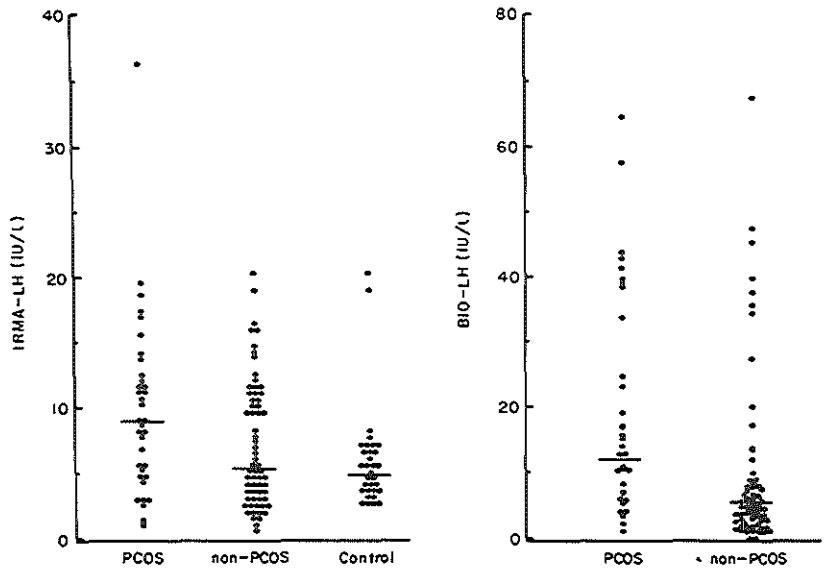


Fig 1. Individual serum IRMA-LH and BIO-LH concentrations in 99 women presenting with infertility and oligo/amenorrhoea with ($n=35$) or without ($n=64$) PCOS, and serum IRMA-LH levels in 30 regularly cycling controls. Bars denote medians.

Results

In the total group of 99 women presenting with infertility and cycle abnormalities 35 patients were diagnosed as PCOS (Table 1), and 42 women exhibited elevated IRMA-LH levels (Table 2). In the total study group a wide range of IRMA-LH and BIO-LH serum concentrations (Fig. 1) was found, with a good correlation ($r=0.66$, $P<0.001$) between these parameters (Fig. 2). There was no significant difference for this correlation of IRMA-LH and BIO-LH in PCOS ($r=0.61$, $P<0.001$) and non-PCOS ($r=0.65$, $P<0.001$) patients. Using logistic regression, the probability of PCOS significantly increases ($P<0.001$) with increasing BIO-LH levels. The probability increase was of marginal significance ($P=0.06$) when determined from IRMA-LH levels. The probability of suffering from PCOS as predicted by BIO-LH or IRMA-LH levels is indicated in Fig. 3. Evaluating both parameters simultaneously, only BIO-LH remained of high significance, while no additional predictive value for PCOS was found for IRMA-LH ($P=0.45$). When the probability was calculated for each patient according to BIO-LH concentrations, there were 21 cases for whom the probability exceeded 50% (12 were diagnosed as PCOS). The same procedure for IRMA-LH led to only 2 cases in whom the probability exceeded 50% (only 1 was diagnosed as PCOS). Correlations between serum T levels and BIO-LH ($r=0.47$, $P<0.001$) and IRMA-LH ($r=0.51$, $P<0.001$) concentrations, and BIO/IRMA-LH ($r=0.15$, $P=0.15$)

IRMA-LH	>8·3 IU/l* ($n=42$)	≤8·3 IU/l ($n=57$)	P value
BMI (kg/m²)	24·0 (18·1–42·2)	23·1 (18·3–48·7)	0·9
Amenorrhoea	52%	33%	0·07
Polycystic ovaries†	60%	40%	0·07
IRMA-LH (IU/l)	11·5 (8·5–35·5)	3·7 (0·3–8·3)	
BIO-LH (IU/l)	16·9 (3·0–67·1)	5·1 (1·0–33·6)	<0·001
BIO IRMA-LH	1·5 (0·2–5·3)	1·3 (0·3–25·8)	0·7
IRMA-FSH (IU/l)	4·5 (1·7–9·3)	4·2 (0·4–10·0)	0·2
IRMA-LH/FSH	2·6 (1·0–10·1)	0·8 (0·2–7·3)	<0·001
T (nmol/l)	2·7 (1·3–7·6)	1·8 (0·6–7·2)	<0·001
FAI	5·8 (1·8–16·7)	3·3 (0·4–20·5)	0·005
AD (nmol/l)	15·1 (5·1–37·0)	10·6 (2·6–33·5)	<0·001
DHEAS (μmol/l)	6·9 (1·8–15·8)	5·8 (0·3–15·6)	0·2
E1 (pmol/l)	385 (233–713)	288 (137–777)	<0·001
E2 (pmol/l)	190 (100–582)	175 (64–783)	0·1

Table 2 Clinical and endocrine characteristics of 99 women presenting with infertility and oligo/amenorrhoea, separated into subgroups according to the presence or absence of elevated IRMA-LH serum levels

* 8·3 IU/l represents mean + 2SD of 30 regularly cycling controls.
† Twelve or more follicles in one ovary, as determined by vaginal sonography.

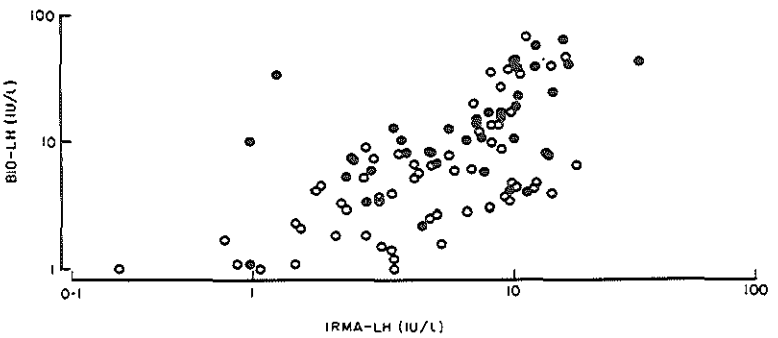


Fig. 2 Correlation ($r=0·66$; $P<0·001$) between BIO-LH and IRMA-LH serum levels in 99 women presenting with infertility and oligo/amenorrhoea. ●, Patients with PCOS.

could be established, either between BMI and follicle number, ovarian volume, and stroma echogenicity, or between BMI and T levels. INS levels were correlated with BMI (r=0.57, P<0.0001), but not with T, I-LH, or BIO-LH.

As displayed in Fig. 1, follicle number, ovarian volume, and stroma echogenicity values were significantly correlated with I-LH (r=0.37, P<0.0001; r=0.30, P=0.003; r=0.29, P=0.005, respectively) and BIO-LH (r=0.37, P<0.0001; r=0.34, P=0.001; r=0.30, P=0.003, respectively) levels. For the same TVS parameters, significant correlations with T (r=0.44, P=0.001; r=0.34, P=0.001; r=0.42, P<0.0001, respectively) concentrations could be established (Fig. 2). FAI correlated also with follicle number (r=0.34, P=0.001), ovarian volume (r=0.24, P<0.003), and ovarian stroma (r=0.27, P=0.009) (data not shown).

Patients (n=95) were divided according to their T and LH levels, and women with

elevated LH and/or T levels were defined as PCOS (n=52), whereas women with LH and T within normal limits were considered as non-PCOS (n=43). Ovarian volume differed between these 2 groups (median 9.8 ml in PCOS versus 7.8 ml in non-PCOS [P<0.05]). The same applied to total follicle number in both ovaries (19 versus 13 [P<0.008]). The percentage of stroma scores ≥2 was higher in PCOS as compared to non-PCOS (94% versus 74% [P<0.01]) cases.

INS and INS-RES were strongly correlated (r=0.99, P<0.0001). Both paramters correlated with the ovarian volume (r=0.24, P<0.03; r=0.23, P<0.05, respectively), and stroma echogenicity (r=0.26, P<0.03; r=0.27, P<0.02, respectively) but not with follicle number. Correlation between ovarian volme and stroma, and INS-RES did not differ significantly between PCOS and non-PCOS cases. INS-RES correlations with sonography parameters are shown in Fig. 2. Using multivariate analysis, INS-RES was found to be of significant (P<0.05) additional predictive value of ovarian volume and stroma echogenicity.

I-LH and T were significantly correlated with each other (r=0.51, P<0.001). Investigating correlations between I-LH and T simultaneously and TVS parameters by multiple regression analysis, it appeared that for none of the TVS parameters the predictive value of T was significantly affected by the levels of I-LH, neither was the predictive value of I-LH affected by the concentration of T. Using the same method of analysis to investigate BIO-LH and T levels together, observations were similar for total number of follicles and stroma. With regard to ovarian volume, there was a significant (P<0.05) predictive value of BIO-LH, whereas T was of only marginal significance (P=0.06). By estimating the relative predictive value of T and I-LH together with regard to all 3 TVS parameters, it appeared for ovarian volume that each time there was a doubling of T value, there was a 23% increase in volume (not significant [P=NS] for I-LH, P=0.03 for T, respectively). For total follicle number, each time there was a doubling of I-LH value, there was a 9% (P=NS) increase in number, whereas each time there was a doubling of T value, there was a 40% (P=0.002) increase in number. For ovarian stroma, on a scale from 1 to 3, each time there was a doubling of I-LH value, there was a 0.1 (P=NS) increase in stroma score, whereas each time there was a doubling of T value, there was a 0.4 (P=0.001) increase in ratios in the total study group are indicated in Fig. 4.

Characteristic of PCOS (based on clinical and endocrine criteria, excluding IRMA-LH) versus non-PCOS patients

Women presenting with PCOS exhibited statistically significant higher BIO-LH, T, AD, and E1 serum concentrations and BIO/IRMA-LH ratios as compared to non-PCOS women (Table 1). Moreover, a tendency towards elevated IRMA-LH (P=0.07), and IRMA-LH/FSH ratios (P=0.06) in the PCOS group was observed. In the PCOS group 18 (51%) women showed elevated IRMA-LH levels, 16 (46%) IRMA-LH/FSH ratios above 2, and 6 (17%) ratios above 3.

Characteristics of women presenting with elevated versus normal LH concentrations

The 42 women presenting with elevated IRMA-LH serum levels exhibited significantly higher BIO-LH, T, FAI, AD, and E1 serum concentrations, and IRMA-

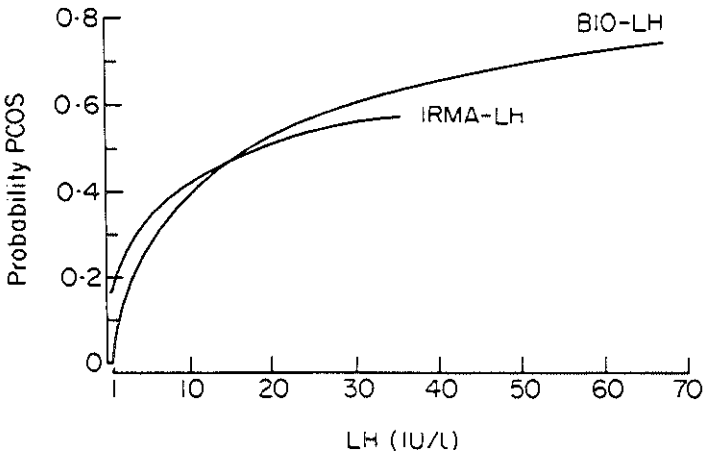


Fig. 3. Estimated probability of PCOS according to levels of BIO-LH and IRMA-LH. Curves are drawn for the observed ranges of LH levels.

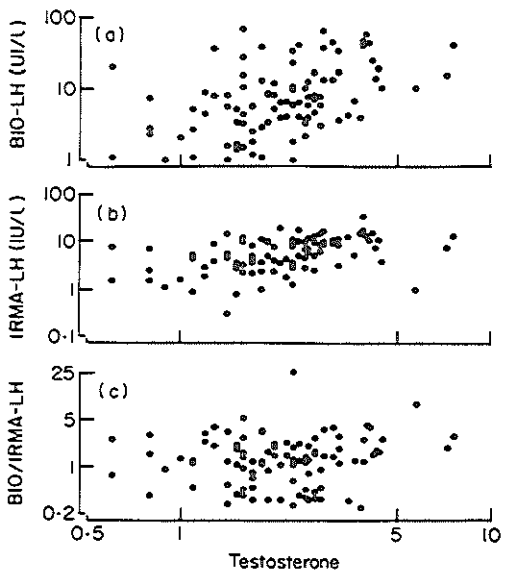


Fig. 4. Correlation between serum testosterone levels and a, BIO-LH (r=0.47; P<0.001) and b, IRMA-LH (r=0.51; P<0.001) concentrations, and c, BIO/IRMA-LH ratios (r=0.15; P=0.15) in 99 women presenting with infertility and oligo/amenorrhoea.

Table 3. Clinical and endocrine characteristics of 99 women presenting with infertility and olio/amenorrhea, separated into subgroups according to high or low BIO-LH concentrations

BIO-LH*	⩽5 (n=36)	5–10 (n=26)	⩾10 (n=37)	P value†
BMI (1kg/m²)	23·0 (18·3–38·5)	22·3 (19·0–32·8)	26·1 (18·1–48·7)	0·09
Amenorrhoea	32%	27%	61%	0·02
Polycystic ovaries‡	42%	42%	62%	0·10
IRMA-LH (IU/l)	3·6 (0·3–15·9)	4·9 (2·4–19·9)	10·4 (1·3–35·5)	<0·001
BIO-LH (IU/l)	2·8 (1·0–4·7)	6·9 (5·1–9·8)	23·8 (10·2–67·1)	
BIO IRMA-LH	0·5 (0·2–2·3)	1·3 (0·3–3·1)	2·4 (0·9–25·9)	<0·001
IRMA-FSH (IU/l)	4·8 (0·5–9·3)	4·4 (0·4–8·3)	4·3 (1·7–10·0)	0·5
IRMA-LH/FSH	0·8 (0·2–3·1)	1·3 (0·5–7·3)	2·5 (0·3–10·1)	<0·001
T (nmol/l)	1·7 (0·6–3·8)	2·0 (0·8–3·6)	2·8 (0·6–7·6)	<0·001
FAI	3·4 (0·4–14·0)	3·3 (1·3–16·5)	6·1 (1·8–20·5)	<0·001
AD (nmol/l)	11·7 (6·1–20·1)	11·5 (7·3–19·6)	15·0 (2·6–37·0)	0·002
DHEAS (μmol/l)	6·2 (1·2–14·3)	5·7 (2·1–15·6)	8·1 (0·3–15·8)	0·1
E1 (pmol/l)	288 (162–696)	301 (137–777)	400 (206–713)	0·001
E2 (pmol/l)	173 (66–746)	156 (64–783)	231 (100–582)	0·002

* Arbitrarily, subdivision was made in groups of roughly similar size.
† Statistical comparison was performed between groups with BIO-LH ⩽5, and ⩾10.
‡ Twelve or more follicles in one ovary, as determined by vaginal sonography.

LH/FSH ratios (Table 2). In addition, a tendency towards the more frequent occurrence of amenorrhea (P=0.07) and polycystic appearance of ovaries (P=0.07) was observed in women with elevated IRMA-LH levels. In this group 25 women (60%) exhibited polycystic ovaries, and only 18 (43%) were diagnosed as PCOS.

Characteristics of women presentinf with high versus low BIO-LH concentrations

The study group was arbitrarily divided in 3 categories (BIO-LH levels above 10, between 5 and 10, and below 5) of roughly similar size (Table 3). Comparing groups with high versus low BIO-LH concentrations, significant differences were observed for IRMA-LH, T, FAI, AD, E1 and E2 serum levels, and IRMA-LH/FSH ratios.

Discussion

In recent literature much attention has been focused on the sonographic diagnosis of polycystic ovaries. It has become evident, however, that a wide overlap with normal ovarian appearance exists (Pache et al. 1990; Pache et al. 1992), and that a proportion of patients with polycystic ovaries may not suffer from the clinical syndrome PCOS (Conway et al. 1989; Eden et al. 1989; Obhrai et al. 1990). Because of the controversy surrounding many features involved in PCOS diagnosis, for clinical practice elevated serum LH concentrations is still considered an important diagnostic feature by many authors. In addition, elevated LH concentrations appear to be involved in reduced success rates of in vitro fertilization and increased risk of early pregnancy loss (Howles et al. 1986; Homburg et al. 1988; Regan et al. 1990).

This study was undertaken to investigate the significance of single serum LH estimates for the diagnosis of PCOS. It should be noted that in the present patient group underweight women were excluded (because of presumed low gonadotropin

levels), and that all patients were suffering from infertility and oligo/amenorrhea. Endocrine abnormalities involved in PCOS may be more pronounced in women presenting with cycle abnormalities as compared to ovulatory women (Eden et al. 1986), and it has recently been shown (Conway et al. 1989) that infertility was associated with elevated serum gonadotropin levels. Since some PCOS patients may have regular cycles and may not present with infertility, conclusions drawn from this study should be restricted to this subgroup only. It should also be emphasized that single measurements of LH may not adequately characterize potential alterations of frequency and/or amplitude of episodic LH secretion. However, a significant proportion of PCOS patients exhibit normal LH levels (Rebar 1984), even if frequent sampling (considering the pulsatile nature of LH release) is applied (Franks 1989). The LH assay used should also be taken into consideration. It has been demonstrated that absolute differences in LH levels comparing PCOS and non-PCOS patients are less pronounced using IRMA as compared to RIA (Fauser et al. 1991b). This may in part be explained by cross reactivity of the RIA with elevated serum levels of α-subunits in PCOS. The quantity of circulating LH in PCOS patients may therefore be substantially overestimated using RIA. On the other hand, the power of LH estimates to discriminate between PCOS and non-PCOS is substantially reduced by using IRMA (Fig. 1). In our previous study (Fauser et al. 1991b), 100% of PCOS patients exhibited LH/FSH ratios above 2 using RIA and only 50% using IRMA. In addition, results may also be influenced by the used IRMA kit (Vermes et al. 1991). It may seem in the present study that IRMA hormone estimates closely resemble LH functional activity, since a good correlation between IRMA-LH and BIO-LH levels was observed. However, the difference in serum levels comparing PCOS versus non-PCOS is much more pronounced for BIO-LH as compared to IRMA-LH (Table 1). Only 43% of women with elevated IRMA-LH levels were diagnosed as PCOS. Moreover, in the total group of women suffering from oligo/amenorrhea BIO-LH levels were found to be a reasonable good predictor of PCOS ($P<0.001$) (Fig. 3), whereas the predictive value was much less for IRMA-LH and no additional predictive value could be observed for IRMA-LH if both parameters were evaluated simultaneously. This suggests indeed, that BIO-LH represents the functional activity of LH involved in the derangement in PCOS.

Dividing the study group into different populations based on PCOS criteria or IRMA-LH levels, yield significant differences in androgen, E1, and BIO-LH levels (Tables 1 and 2). The most striking observation is the relative small difference in IRMA-LH levels between both groups resulting in different BIO/IRMA-LH ratios comparing PCOS versus non-PCOS. This indicates that alterations in LH biopotency (BIO/IRMA-LH ratios) in PCOS depend on differences in BIO-LH, rather than IRMA-LH concentrations. Taken together, this favors the concept of circulating LH isohormones with high biological activity in PCOS patients, as suggested previously (Fauser et al. 1991b). Comparison between women presenting with high versus low LH biopotency demonstrate that endogenous steroid concentrations including E1, E2, AD, FAI, DHEAS (data not shown) and T (Fig. 4) do not correlate with BIO/IRMA-LH ratios. This is contradictory to observations after orchiectomy or following T

administration in hypogonadal men (Jaakolla et al. 1990; Tsatsoulis et al. 1990).

It can be concluded from the present study that women suffering from infertility and oligo/amenorrhea, classified by the presence or absence of signs of PCOS or elevated IRMA-LH levels exhibit different clinical and endocrine characteristics. PCOS is characterized by elevated LH biopotency, and BIO-LH serum levels were found to be a good predictor of PCOS. In contrast, only a minor difference was observed for IRMA-LH concentrations comparing PCOS versus non-PCOS patients.

Summary

Objective: Evaluation of the significance of single serum LH estimates (as assessed by radiometric assay [IRMA] and Leydig cell in vitro bioassay [BIO]) for the diagnosis of polycystic ovary syndrome (PCOS) in women with infertility and cycle abnormalities.
Design: Hormonal and clinical comparisons between subgroups were made based on classification according to: a)rigid clinical and endocrine (excluding LH) characteristics of PCOS, b) elevated IRMA-LH concentrations, c) BIO-LH levels. In addition, androgen modulation of LH biopotency was studied in these patients.
Patients: 99 women presenting at our Infertility Unit with oligo/amenorrhea.
Measurements and results: In the total study group 35 women were diagnosed positive as PCOS and 42 showed elevated IRMA-LH levels. Only 51% (n=18) of PCOS patients showed elevated IRMA-LH levels, and in PCOS significantly higher concentrations of BIO-LH, androstenedione, estrone and BIO/IRMA-LH ratios were found as compared to non-PCOS patients. In the group with elevated IRMA-LH levels only 43% (n=18) was diagnosed as PCOS, and no difference in BIO/IRMA-LH ratios was found. With increasing BIO-LH levels the probability of PCOS rises sharply ($P<0.001$), whereas this probability is of only marginal significance ($P=0.06$) for IRMA-LH. In the total study group a correlation is observed between serum testosterone (T) levels and IRMA-LH ($r=0.47$), and BIO-LH ($r=0.51$) concentrations. This correlation is absent comparing T and BIO/IRMA-LH ratios ($r=0.15$).
Conclusions: Results presented in the present study indicate: 1) women with infertility and oligo/amenorrhea classified based on signs of PCOS or IRMA-LH levels, exhibit different clinical and endocrine characteristics, 2) only 51% of PCOS women exhibit elevated IRMA-LH concentrations, and only 43% of women with elevated IRMA-LH were diagnosed as PCOS, 3) IRMA-LH levels are a poor predictor of PCOS, whereas the predictive value of BIO-LH is better, 4) elevated BIO/IRMA-LH ratios in PCOS are dependent on alterations in BIO-LH, rather than IRMA-LH concentrations, 5) no correlation was observed between serum T levels and BIO/IRMA-LH ratios.

7.4 Association between ovarian changes assessed by transvaginal sonography and clinical and endocrine signs of the polycystic ovary syndrome

Introduction

Assessment of ovarian morphology by means of ultrasound is currently employed as a substitute for histologic examination in the diagnosis of polycystic ovaries. Validity of transvaginal sonography (TVS) to assess ovarian volume and stroma, and follicle number, has been demonstrated in women with regular (Pache et al. 1990), or irregular cycles (Pache et al. 1991a), and reference values to discriminate between normal and polycystic ovaries have been reported (Pache et al. 1992a). However, because of the heterogeneity of the polycystic ovary syndrome (PCOS), ultrasound can not be utilized alone for diagnosis. It should therefore be examined to what extent ascertainment of ovarian changes by TVS may contribute to the diagnosis of PCOS based on endocrine and clinical observations.

Elevated immunoreactive luteinizing hormone (I-LH) and/or androgen levels, next to hyperinsulinemia and insulin (INS) resistance, are believed to be crucial factors involved in maturation arrest of follicles in polycystic ovaries. It has also been shown (Lobo et al. 1983; Fauser et al. 1991b) that abnormally high bioactive LH (BIO-LH) serum levels are a distinct feature in PCOS women. Sonography examination may allow to assess whether the magnitude of ovarian polycystic transformation preferentially corresponds to BIO-LH concentrations as compared to I-LH. Hyperandrogenemia is a classical key feature of PCOS (Goldzieher & Axelrod 1963), and could in part result from hyperinsulinemia or INS resistance (Burghen et al. 1980; Nestler et al. 1989b; Poretsky 1991). INS resistance is also commonly associated with obesity, a frequent finding in PCOS women. If high androgens or INS resistance play a major role in the pathogenesis of PCOS, these parameters should also correlate with polycystic changes of the ovaries. The objective of the present study was to investigate whether ovarian changes - as estimated by TVS - would reflect coincidental alterations of clinical and endocrine findings commonly asociated to PCOS.

Materials and Methods

Subjects and study protocol

The protocol was approved by the local Ethics Review Committee. Ninety-five patients aged 27 $\pm$ 5 years (mean $\pm$ SD) attending the Infertility Clinic participated in the study after informed consent was obtained. All patients presented with infertility (primary=62, secondary=33) of more than 1 year duration. Their mean weight was 25.7 kg/m^2(body mass index [BMI]). Underweight women (BMI <19 kg/m^2) were not included in the study, because of presumably low serum gonadotropin levels. Hirsutism was defined using the Ferriman & Gallwey (F-G) score (1961). Fifty-eight (61%) patients were oligomenorrheic (cycle length $\geq$ 35 days), and 37 (39%) were

amenorrheic (no vaginal bleeding for at least 6 months).

Patients were studied in the follicular phase before cycle day 12 in oligomenorrheic women, or at random in amenorrhea. Ultrasound examination of the ovaries was performed by the same observer (T.D.P.) using a 5 MHz vaginal transducer (Orion; Philips Medical Systems, Eindhoven, The Netherlands), according to a method described previously (Pache et al. 1990). Ovarian volume and follicule number were recorded. Ovarian stroma echogenicity was scored as 1 (normal), 2 (moderately increased), or 3 (markedly increased) (Pache et al. 1991a). Peripheral blood was obtained from 95 fasting patients between 8:00 and 11:00 AM, the same day ultrasound examination took place. To the exception of the blood kept for glucose estimation, samples were centrifuged (10 minutes, 2800 G), and serum was stored at -20°C until assayed.

Hormone estimations

Serum was assayed for I-LH, BIO-LH, testosterone (T), and sex hormone binding globulin (SHBG). The ratio of Tx100/SHBG was used to calculate the free androgen index (FAI). Kits for measurement of LH by radioimmunometric assay (IRMA) were purchased from Medgenix (Fleurus, Belgium), as previously described (Fauser et al. 1990). Intra- and interassay coefficient of variation (CV) was less than 5 and 15%, respectively. BIO-LH levels were measured using the in vitro Leydig cell bioassay (Van Damme et al. 1974). As previously mentioned (Fauser et al. 1991b), the bioassay standard curve was parallel for the IRMA standard (78/549) and for LER-907. All LH bioassays were run in the same assay, with an intrassay CV of 12%. T was determined by radioimmunoassay (RIA) as described previously (van Landeghem et al. 1981), and the intra- and interassay CV was less than 3 and 5%, respectively. SHBG RIA kits were obtained from Diagnostic Products Corp. (Los Angeles, CA, USA). The intra- and interassay CV was less than 4 and 5 %, respectively. 8.2 IU/L was considered to be the upper normal limit for I-LH and 2.6 nmol/L for T, based on hormonal estimates in 29 regularly cycling women (Pache et al. 1992a).

An automatic hexokinase method was used to measure fasting glucose (GLU) levels in venous whole blood. Fasting INS was estimated by enzyme-linked immunosorbant immunoassay (ELISA) obtained from Medgenix. Intra- and interassay CV was less than 6 and 10%, respectively. Insulin resistance was assessed with the formula: INS-RES= (INS x GLU)/7 x 22.5 (Matthews et al. 1985). Since INS was estimated in 86 patients, and GLU in 75, INS-RES could be calculated in 75 women only.

Statistical analysis

Comparisons of categorical data between groups were done with the Chi-square test. Continuous data were compared by using the Mann-Whitney'y test. Hormonal data and TVS parameters such as follicle number and ovarian volume values were logarithmically transformed to reduce skewness of ditributions. Simultaneous evaluation of correlations between INS levels, INS-RES, and sonography parameters (such as ovarian volume and stroma echogenicity, and follicle number) in PCOS and non-PCOS patients was performed by multiple regression analysis. Correlation coefficients given

are Pearson. $P<0.05$ was taken as the limit of statistical significance. Data are presented as mean $\pm$ SD unless otherwise indicated.

Results

Sixty-nine patients (63%) presented with a F-G score ≥ 8, and were therefore considered as hirsute. There was a significant correlation between the F-G score and the number of follicles, the ovarian volume and stroma echogenicity ($r=0.33$, $P=0.001$; $r=0.49$, $P<0.001$; and $r=0.39$, $P<0.001$, respectively [data not shown]). No relationship stroma echogenicity.

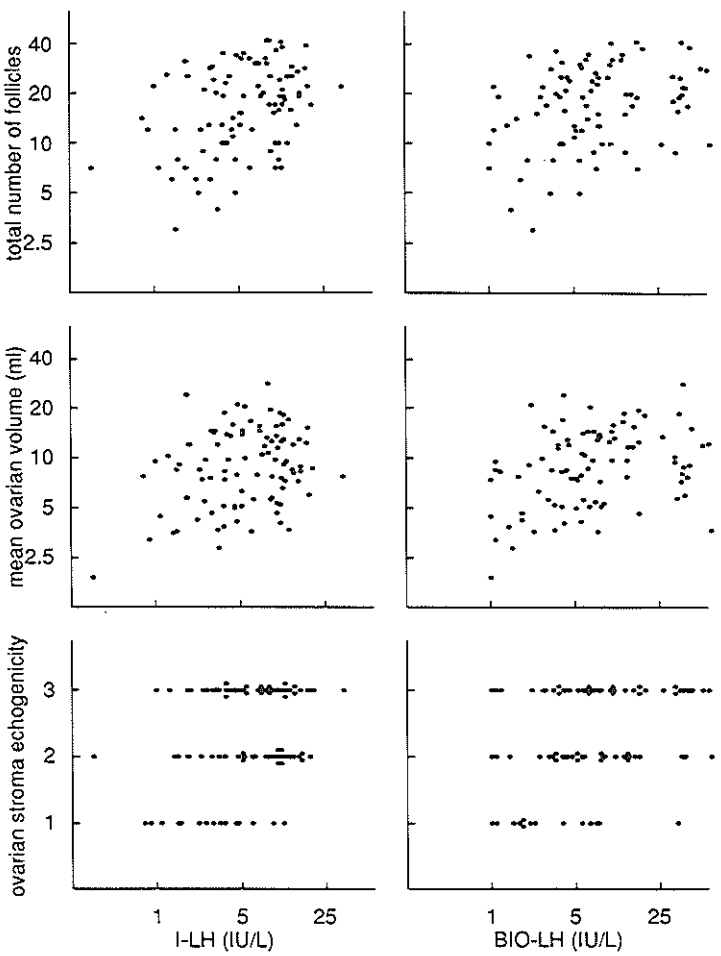


Figure 1
Correlations between I-LH and BIO-LH and trans-vaginal sonography features (follicle number [upper panel], ovarian volume [middle panel] and ovarian stroma echogenicity [lower panel] in 95 women with infertility and cycle disturbance.

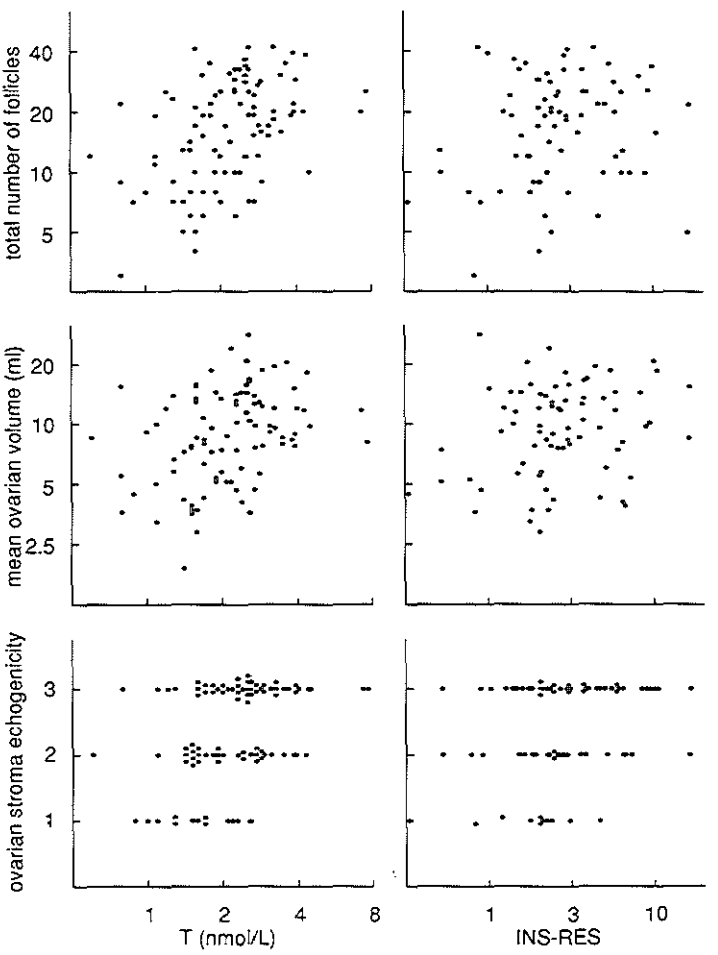


Figure 2
Correlations between T and INS-RES and transvaginal sonography features (follicle number [upper panel], ovarian volume [middle panel], and ovarian stroma echogenicity [lower panel] in 95 women with infertility and cycle disturbance.

Discussion

The present study demonstrates that unusually high I-LH, BIO-LH, and T levels, are associated with abnormally high numbers of small sized follicles, with an increase in the amount of ovarian stroma and with augmented ovarian volume. All these sonographic parameters are criteria of polycystic transformation of ovaries (Adams et al. 1985; Pache et al. 1992a). The observation that endocrine characteristics of PCOS coexist with classical morphological transformation of this disease (Hughesdon 1982) detected by ultrasound examination provides evidence that monitoring of the ovaries by sonography can be used as an additional tool for the diagnosis of the syndrome.

Since it was shown that elevated BIO-LH is a distinct feature of PCOS women (Lobo et al. 1983; Fauser et al. 1991b) and correlates more closely with PCOS as compared to I-LH (Fauser et al. 1992a), we investigated whether the degree of correlation between LH bioactivity and magnitude of ovarian changes would be better as compared to I-LH. Data from the present work do not provide evidence that this is the case, although correlation between ovarian stroma and volume was slightly higher with BIO-LH than with I-LH. Abnormally high BIO-LH levels supposingly stand for the dysfunction of LH secretion in the pathogenesis of PCOS (Fauser et al. 1992a). The notion that excessive LH bioactivity is not better reflected in polycystic alterations of ovarian architecture than I-LH, may underscore the limited role of this glycoprotein in the genesis of the disorder in a proportion of women. Besides, T correlated better than BIO-LH with all sonography parameters. Up to now, results from the present study seem to confirm that elevated LH levels are not a prerequisite for polycystic transformation of the ovaries, as recently demonstrated in long term androgen treated female to male transsexuals (Pache et al. 1991c). In this regard, it should be noted that up to 40% of women with clinical and endocrine signs of PCOS exhibit I-LH levels within normal limits (Fauser et al. 1992a). Altogether, these observations may indirectly favor the concept of intra-ovarian dysregulation as a major cause of polycystic transformation of the ovaries (Pache & Fauser 1991b; Erickson et al. 1992).

Hirsutism - a salient feature of PCOS - was present in 63% of the patients, which is similar to the previously reported incidence of this sign in PCOS (Goldzieher & Axelrod 1963; Franks 1989). Hirsutism was positively correlated with all ultrasound features of polycystic ovaries. Association between total and free T, and raised numbers of small follicles and increase stroma echogenicity came as no surprise, since it has been shown that androgens alone can induce polycystic transformation of ovaries in the human (Pache et al. 1991c). Since T, I-LH, and BIO-LH levels all correlated with sonographic parameters, it should be noticed that gonadotropin and T levels were independently correlated with the ovarian structure. Moreover, when the predictive value for ovarian structure of I-LH and T together was assessed, there was no significant additional predictive value for I-LH. The same observation applied to BIO-LH and T. Again, these observations emphasize the apparent instrumental role of androgens in the genesis of polycystic ovaries, and additionally support the use of sonography in the diagnosis of PCOS.

Hyperinsulinemia and INS resistance have been proposed as major determinants of hyperandrogenemia (Poretsky 1991) in both lean and obese PCOS women (Chang et al. 1983; Dunaif et al. 1987). However, up to now studies were performed only in women with clinical and endocrine evidence of PCOS. It remained to be investigated whether INS resistance could be found in all PCOS women with morphological evidence of polycystic ovaries. In the present work, INS and INS-RES were positively associated with ovarian volume and stroma echogenicity, and INS-RES could significantly predict ovarian volume and stroma amount. Androgens are synthesized in stroma and theca cells. An increased rate of atresia - with consecutive augmentation of ovarian volume - is presumably part of the mechanisms leading to polycystic

transformation of ovaries (Pache et al. 1991c). This concept together with our findings related to INS and INS-RES confirm previous reports making INS a likely candidate as a stimulator of ovarian androgen production (for review, see Poretsky 1991). However, we were unable to demonstrate a correlation between INS and T levels, irrespective of whether the analysis was made in the whole group, or in PCOS and non-PCOS subgroups. This is at variance with most previously published reports (Poretsky 1991), but in keeping with recent work from Sharp and colleagues (1991). This may suggest that INS may be only indirectly involved in increase androgen production at the ovarian level. Moreover, INS-RES did not differ between PCOS and non-PCOS cases, whereas INS levels correlated with BMI but not with T. Since BMI did not differ between PCOS and non-PCOS women (data not shown), this could mean that INS resistance is preponderently dependent on weight, and is not an overall common denominator of polycystic ovarian changes.

In conclusion, observations from the present study, in combination with other studies reported by our group (Pache et al. 1991a; Fauser et al. 1991b; Pache et al. 1992a; Schoot et al. 1992b), and by others (Ardaens et al. 1991) indicate that transvaginal ultrasonographic assessment of ovaries can be a valuable tool for the diagnosis of PCOS. T levels are better predictors of ovarian structure than I-LH and BIO-LH are, and the presence of INS resistance may be considered as an additional predictor of ovarian volume and stroma amount. In addition, the LH-independent effect of androgens on polycystic ovarian changes is highlighted. This may further substantiate the important role of androgens in the pathophysiological mechanisms of PCOS.

Summary

Objective and design: a prospective study was designed to determine whether ovarian polycystic changes estimated by transvaginal sonography correlate with clinical and endocrine findings associated to the polycystic ovary syndrome.

Setting: reproductive endocrinology unit in the Department of Obstetrics and Gynecology at a teaching hospital.

Participants: ninety-five consecutive patients entering an infertility treatment program.

Interventions: all women were examined by transvaginal sonography, assessed for body mass index and hirsutism. Blood withdrawal was performed for hormone estimates.

Main outcome measures: ovarian follicle number, volume and stroma echogenicity. Immunoactive and bioactive LH, T, free T (FAI), insulin (INS) levels and resistance index (INS-RES) measurements were performed.

Results: hirsutism was present in 63% of the patients, and correlated with the number of follicles, the ovarian volume and stroma echogenicity ($r=0.33$, $P=0.001$; $r=0.49$, $P<0.001$; and $r=0.39$, $P<0.001$, respectively). Follicle number, ovarian volume,

and stroma echogenicity values were significantly correlated with I-LH (r=0.37, P<0.0001; r=0.30, P=0.003; r=0.29, P=0.005, respectively), BIO-LH (r=0.37, P<0.0001; r=0.34, P=0.001; r=0.30, P=0.003, respectively), T levels (r=0.44, P=0.001; r=0.34, P=0.001; r=0.42, P<0.0001, respectively), and FAI (r=0.34, P=0.001; r=0.24, P<0.003; r=0.27, P=0.009, respectively). Both T and I-LH were independently correlated with ovarian structure TVS parameters. INS and INS-RES correlated with ovarian volume (r=0.24, P<0.03; r=0.23, P<0.05), and stroma echogenicity (r=0.26, P<0.03; r=0.27, P<0.02). INS-RES was of significant (P<0.05) additional predictive value of ovarian volume and stroma amount. Evaluating the predictive value of I-LH and T together in regard to all TVS parameters, only T levels were statistically significant predictors of increase in follicle number, ovarian volume and stroma amount. The same observation applied for BIO-LH and T together in respect to follicle number and stroma echogenicity, whereas for ovarian volume BIO-LH was a better predictor than T.

Conclusions: transvaginal sonography assessment of ovaries can be a valuable tool for the diagnosis of PCOS. INS resistance is of additional predictive value of ovarian volume and stroma echogenicity. The independent effect of androgens on ovarian changes further substantiate their apparent cardinal role in the genesis of polycystic ovaries.

7.5 Ovarian follicular fluid mirrors granulosa cell regulation and function

Follicles are the basic functional units in the ovary (Hsueh et al. 1984), and a definite succession of hormonal changes occur within these microenvironments, changes which are crucial in determining their development (McNatty et al. 1975). Intra-follicular concentration of steroids and gonadotropins correlate better with mitotic and biosynthetic activities of the granulosa cells than do plasma hormone levels (McNatty et al. 1979), thus providing a most accurate image of ovarian physiology. Consequently, investigation of follicle growth regulation implies measurements at the functional unit level. Antral follicles in diverse stages of development - healthy or atretic - are present during the entire menstrual cycle, and one way to assess their microenvironment in vivo is to puncture fluid from their cavities. Accordingly, hormonal estimates (oestradiol, androstenedione, inhibin) were performed in ovarian follicle fluid obtained from women with regular cycles, and from women with polycystic ovaries. In an earlier chapter of this thesis (chapter 5), female to male transsexuals have been proposed as a human model for the study of polycystic ovaries. Hence, follicular fluid was also obtained from this group of women.

One major limitation in this kind of studies is the limited amount of fluid which could be collected: a 3.0 mm follicle will contain only 14 ul of fluid, only 110 ul fluid will be collected from a 6.0 mm follicle (for calculation, volumes in spheres were considered, $4/3 \times \pi_r^3$). This ideal amount is never obtained because granulosa cells also account for part of the volume. In addition, one should not try too hard to withdraw

as much fluid as possible from one follicle, since blood contamination of the sample will render it unusable for hormonal assays.

7.6 17-ß oestradiol, androstenedione, and inhibin levels in fluid of individual follicles of normal and polycystic ovaries, and in ovaries from androgen treated female to male transsexuals

Introduction

In women affected by the polycystic ovary syndrome (PCOS), much evidence has been accumulated that androgens play a key role in mechanisms involved in follicular growth failure. Their ability to induce atretic changes in ovarian follicles has been demonstrated in androgen-treated female to male transsexuals (TSX) (Pache et al. 1991a). Besides, abnormally high androstenedione (AD) levels have been found in pooled ovarian follicular fluid (Short & London 1961, Giorgi 1963) and tissue (Lanthier & Sandor 1960, Mahesh & Greenblatt 1962, Axelrod & Goldzieher 1962) of polycystic ovaries. Under most reported conditions (Rebar et al. 1976, Rosenfield et al. 1990, Fauser et al. 1991), an increased production of androgens per follicle-unit should be found. Alternatively, it could be hypothesized that elevated androgens are merely due to the increased number of androgen-producing units, e.g. cystic atretic follicles. Would this happen to be true, androgen production per follicle unit should not be different in normal and in polycystic ovaries.

As suggested by in vitro (Erickson et al. 1979, Mason et al. 1990) and in vivo studies (Short & London 1961, Eden et al. 1990), absence of selection of one dominant follicle in polycystic ovaries might be due to deficient follicle stimulating-hormone (FSH)-induced granulosa cell aromatase activity, resulting in diminished production of oestradiol (E2). Should follicular maturation arrest in polycystic ovaries represent disturbance of selection, one may hypothesize that a proportion of the follicular population up to a size of 10 mm could still possess the characteristics of developing follicles.

Within the ovary, inhibin (INH) is produced by granulosa cells exclusively, and has been proposed as a non-steroidal marker of granulosa cell function and follicular viability (Channing et al. 1981, McLachlan et al. 1987). In this regard, INH could be considered as an additional tool to compare granulosa cell populations. Besides, bioactive INH was reported to be elevated in polycystic ovaries (Tanabe et al. 1983), and could therefore play a role in granulosa cell dysfunction.

Given the above, follicular fluid has been obtained from ovaries of women with regular cycles, of subjects with polycystic ovaries, and of polycystic ovaries from long-term androgen treated TSX patients, and assayed for AD, E2, and INH. The major objective of the study was to appraise in vivo the ovarian steroidogenic function of individual follicles in polycystic ovaries

Subjects and methods

The study protocol was approved by the Ethics Review Committee of the Dijkzigt Academic Hospital/Erasmus University, and informed consent was obtained from each patient.

Women with regular menstrual cycles

Sixteen women volunteered to participate in the study. Their mean age was 33 years (range 27 to 44 years). All these women were regularly menstruating with a mean cycle length of 28 ± 2 (mean ± SD) days. Mean body mass index (BMI) was 23 ± 3 kg/m^2. In all 16 women, a laparotomy was performed between cycle day 1 and 12, either for refertilisation (n=12), or for surgery of the adnexae (n=4). Peripheral blood was drawn at the same day before surgery took place. After centrifugation (2000 G x 10 minutes), plasma was stored at -20°C.

Two methods were used to collect follicular fluid. In 9 of 16 cases, each follicle bulging at the ovarian surface was measured, then punctured in situ. The technique used to collect follicular fluid in the other 7 subjects was ultrasonography. A real-time Brüel & Kjaer Ultrasound Scanner (Copenhagen, Denmark) equipped with a 7 MHz sector transducer (Type 8538) was used. During laparotomy, the tip of a sterile probe was applied directly against the ovary, and follicles were punctured in situ after measurement. In 3 ovaries, surface follicles were damaged during adhesiolysis, and no fluid was obtained. Follicular fluid from every follicle was kept separately. Although most collected samples were immaculate, centrifugation at 2500 G for 10 minutes was performed, and supernatant kept frozen at -20°C until assayed.

Women with polycystic ovaries

Five infertile women, 4 with amenorrhea and 1 with severe oligomenorrhea (cycle length 72-84 days) underwent transvaginal sonography examination. All 5 fulfilled our criteria for sonographic diagnosis of polycystic ovaries: (1) ovarian volume above 8.0 ml, (2) > 11 follicles between 2 and 10 mm in size in each ovary, and (3) increased ovarian stroma echogenicity (Pache et al. 1991b). Mean age was 28 years (range 24 to 32 years). 2 women were obese (BMI > 25 kg/m^2) and hirsute (Ferriman and Gallwey score [F-G] > 8 (1961), 1 woman was hirsute only.

Peripheral blood withdrawal was performed on the same day ultrasound examination took place, centrifuged (2000 G x 10 minutes), and plasma was stored at -20°C. In one woman, follicles were punctured in situ during laparatomy performed to remove a coexistent dermoid cyst. In the other 4 women, follicular fluid aspiration was performed under transvaginal sonography guidance, on an out-patient basis. In the oligomenorrheic woman, follicle puncture took place 76 days after the last menstrual period. In 3 ovaries, follicular fluid could not be adequately harvested. Follicular fluid was handled as described above for controls.

Female to male transsexuals

Fourteen women with gender dysphoria underwent hysterectomy and bilateral oophorectomy. Mean age at time of surgery was 26 years (range 18 to 35 years). Menarche was normal and menstrual cycles were regular before androgen therapy was

started in all women. Testosterone (T) treatment was given for a median period of 24 months (range 12 to 72) before surgery took place. Two types of T were used for pre-operative treatment. 250 mg Sustanon (Organon, Oss, The Netherlands) (a mixture of 30 mg T propionate, 60 mg T phenylpropionate, 60 mg T isocapionate, and 100 mg T undecanoate), was given im every other week for a median period of 15 months (range 12 to 72 months) to 8 patients. Andriol (Organon, Oss, The Netherlands) is T undecanoate, and was given orally, 120-160 mg/day for a median period of 19 months (range 12 to 25 months) to 6 patients. In all patients, T medication was discontinued 1 month before surgery.

Peripheral blood was drawn before the intervention, centrifuged (2000 G x 10 minutes), and plasma was stored at -20°C. In the operating room, immediately after oophorectomy had taken place, follicle puncture was performed as reported above in controls. In 5 ovaries, no fluid could be obtained. Either were follicles to small to be punctured, or the ovary had been damaged during surgery. Follicular fluid was handled as described above for controls.

Hormonal assays

Immunoreactive serum LH and FSH levels were determined by using a commercially available radio-immunometric (IRMA) kit (Medgenix, Fleurus, Belgium) (Fauser et al. 1990). T in serum was measured by radioimmunoassay (RIA) as described by Verjans et al. (1973). Serum E2 was measured by RIA (Diagnostics Products Corp.). Serum AD was measured using the antiserum described by Frölich and co-workers (Frölich et al. 1976), after extraction of serum with diethyl ether. Intra- and interassay coefficients of variation were less than 5 and 15% for LH, less than 3 and 8% for FSH, less than 3 and 5% for T, less than 5 and 8% for E2, and less than 7 and 15 % for AD, respectively.

Follicular fluid was assayed for E2, AD, and INH content. E2 and AD were measured as indicated above in serum. E2 levels were assayed in 2 dilutions (1:100, and 1:1000), and no deviation from parallelism was observed. INH immunoreactivity was assayed using the antiserum against bovine INH (no. 1989) described by Robertson and colleagues (Robertson et al. 1988), using radio-iodinated 32 kDa bovine INH as a label (Monash University, Department of Anatomy, Melbourne, Australia). These materials were kindly provided by Dr G. Bialy, NICHHD, Bethesda, MD. INH was labeled using [^{125}I]sodium iodide (Amersham, Amersham, United Kindgom) and Protag (Baker, Deventer, The Netherlands). Labeled protein was isolated after chromatography on prepacked Sephadex G-25 columns (PD-10, Pharmacia, Uppsala, Sweden). The immunoassay standard was a bovine follicular fluid preparation with an arbitrary potency of 1 U/µg protein (Grootenhuis et al. 1990). Expressed in units of this standard, the International Research Standard for INH (86/890) has a specific activity of 60 $\pm$ 10 U/µg. All follicle fluid INH levels were estimated in one assay. The intra-assay coefficient was 17.5%

Data analysis

According to criteria established by McNatty and co-workers (McNatty et al. 1979c; McNatty et al. 1981), only individual follicles in which the AD/E2 ratio was

≤ 4 were regarded as healthy. Follicles in which this ratio was above 4 were considered as going into atresia. AD, E2, and INH content was analyzed only in presumably non-dominant follicles (≤ 10 mm in size). Because of blood contamination, fluid from all follicles aspirated from a particular patient were not always available for hormonal analysis.

Comparisons of serum hormone levels between the 3 groups were performed with the Kruskal-Wallis test. In case this test indicated significant differences at the 0.05 % level, pairwise comparisons were made by using the Mann-Whitney test. Since distributions of the intra-follicular levels of AD, E2, INH, and of the AD/E2 ratio were skewed, geometric means of the mean concentration values have been calculated. Because all follicle fluid parameters showed significant between-women differences, Mixed Model Analysis of Variance (BMDP 1983) was used to evaluate between-group differences, with random subject effects nested within groups.

Results

Women with regular menstrual cycles

Values of LH, FSH, and T, are exposed in Table I. During the first 12 days of the cycle, 120 follicles ≤ 10 mm were punctured in 29 ovaries from 16 women with regular cycles. Their mean size was 6.0 mm (range 2.0 to 10.0 mm). Geometric mean levels of E2, AD, and INH values, and calculated AD/E2 ratios in fluid of all follicles together were 91 nmol/L, 3160 nmol/L, 741 U/ml, and 35, respectively. At the time of follicle puncture, one follicle > 10 mm could be observed in 11 out of the 16 regularly cycling women. In addition to their larger size, all these 11 follicles had a AD/E2 ratio far below 4 (data not shown), and were therefore considered as dominant follicles. On average, 12% of the punctured follicles were healthy. Variations in this percentage of healthy follicles, and intra-follicular E2, AD, INH levels, were related, neither to the day of the menstrual cycle, nor to the presence or absence of a coexistent dominant follicle.

Women with polycystic ovaries

Serum values of LH, T, and FSH are presented in Table I. Median AD in serum was high (17.7 nmol/L; range 13.1 to 33.5 nmol/L). 43 follicles were punctured in 7 ovaries. Their mean size was 6.4 mm (range 4.5 to 9.0 mm). Geometric mean levels of E2, AD, and INH values, and calculated AD/E2 ratios in fluid of all follicles together were 48 nmol/L, 3240 nmol/L, 525 U/ml, and 68, respectively. The percentage of healthy follicles was 17%.

Female to male transsexuals

Post-treatment values (just before surgery) of LH, T, and FSH are exposed in Table I. All 14 TSX patients were amenorrheic, and displayed a male phenotype, after a median period of 17 months (range 12 to 72 months) of androgen treatment. 120 follicles were punctured in 23 ovaries, their mean size was 5.0 mm (range 2.0 to 10.0 mm). Geometric mean levels of E2, AD, and INH values, and calculated AD/E2 ratios in fluid of all follicles together were 33 nmol/L, 2630 nmol/L, 490 U/ml, and 80,

respectively. On average, 14% of the follicles were healthy.

Comparisons between groups

Serum values of LH, T, and FSH in controls, PCOS, and TSX, are indicated in Table I. FSH values were not different between groups. Median LH concentration was higher in PCOS than in controls, but the difference did not reach the level of statistical significance. Median LH was lower in TSX than in controls ($P<0.05$), and higher in PCOS than in TSX ($P<0.01$). As compared to controls, median T was higher in PCOS and in TSX (both cases, $P<0.01$). No statistically significant difference could be established in levels of T between PCOS and TSX.

Table 1 Serum levels of LH, FSH, and T in 16 regularly cycling women (C) between cycle day 1 and 12, five women with polycystic ovaries (PCO), and 14 transsexual (TSX) subjects. Values correspond to median and range

	LH (IU/l)	FSH (IU/l)	T (nmol/l)
C	6·8 (2·3–22·0)	5·1 (2·8–11·7)	1·6 (0·8–2·4)
PCO	10·6 (7·9–12·1)	6·9 (3·9–8·3)	2·9 (1·6–7·2)
TSX	2·6 (0·1–10·5)	4·7 (0·4–9·4)	2·7 (0·8–30·2)

In 16 control women, in 5 PCOS patients and in 14 TSX subjects, between-patient differences in mean values of AD (Fig. 1), E2, INH, and AD/E2 ratios (not shown) in follicular fluid were significantly larger than could be expected in view of the variation between follicles within individuals. The geometric mean values of intrafollicular E2, AD, INH, and the AD/E2 ratios in all three groups are displayed in Fig. 2. Taking between-patient differences into account, no significant differences could be established between the three groups for these parameters in follicles $\leq$ 10 mm.

On average, the percentage of healthy follicles did not differ significantly between groups. The size of punctured follicles differed between the 3 groups ($P<0.02$). Mean size of follicles was smaller in TSX (5.0 mm), as compared to controls (6.0 mm) ($P<0.05$), and to PCOS (6.4 mm) ($P<0.01$). In all three groups, no correlation could be established between intra-follicular E2, AD, INH, AD/E2, and follicle size, or serum E2, FSH and LH levels (data not shown). In none of the 3 groups could the proportion of healthy follicles be related to peripheral gonadotropin levels. Intra-follicular steroid values were not related to BMI of the women.

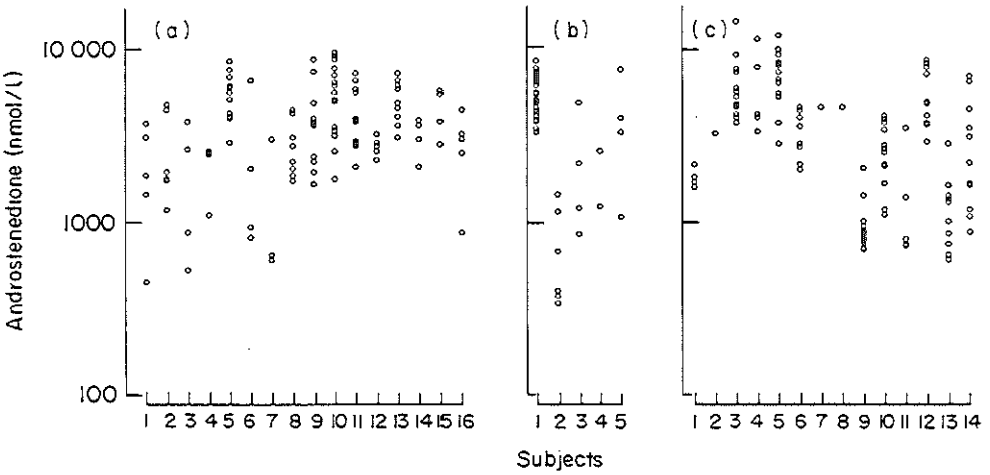


Fig. 1 Androstendione concentrations in individual follicles ≤ 10 mm of a, 16 regularly cycling women (controls) collected between cycle day 1 and 12; b, five women with polycystic ovaries (PCO); and c, 14 transsexual (TSX) subjects.

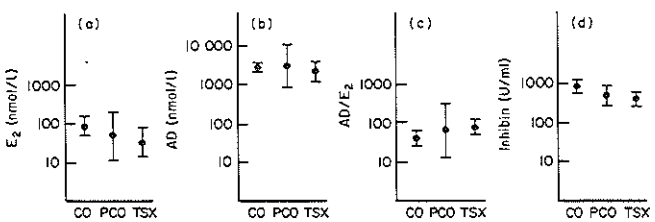


Fig. 2 Comparison of intra-follicular levels of a, E_2 ($P = 0{\cdot}42$); b, AD ($P = 0{\cdot}67$); c, AD/E_2 ratios ($P = 0{\cdot}18$); and d, INH concentrations ($P = 0{\cdot}14$), between 16 regularly cycling women (CO), five women with polycystic ovaries (PCO), and 14 transsexual (TSX) subjects. Values are geometric means (95% confidence intervals) in follicles ≤ 10 mm. Follicles of CO women were collected between cycle days 1 and 12. *P* values denote the absence of statistically significant differences between groups.

Discussion

Both PCOS and TSX groups presented with serum androgen levels higher than in controls, whereas TSX subjects differed from PCOS patients by their reduced LH levels consecutive to T-induced negative feedback activity at the hypothalamo-pituitary level (Spinder et al. 1989). Ovarian polycystic changes induced by exogenous androgens (Pache et al. 1991a) are similar to those observed in women presenting with elevated endogenous androgen production and diminished LH secretion (Erickson et al. 1989). Altogether, this may not only suggest that androgens in excess are instrumental in the induction of ovarian abnormalities, but also that a high LH concentration is not required for polycystic ovarian transformation.

Measurements of AD and E2 in follicular fluid confirmed that, in normal ovaries, each follicle has a different steroid profile (McNatty et al. 1979c). In addition, INH levels were also found to vary between follicles within one individual. Our results indicate that steroid and INH levels differ also in follicles harvested from polycystic ovaries of PCOS and TSX patients. Ascertainment of wide differences in steroids and INH levels between women in all three groups was a striking finding, which precluded pooling of follicular fluid. Former studies with pooled human follicular fluid have reported abnormally high levels of AD and low or undetectable E2 in polycystic ovaries (Short & London 1961, Giorgi 1963, Jeffcoate et al. 1968). Our data do not confirm these observations, and also show that E2 could be detected in follicles as small as 2 mm in all 3 groups. Moreover, taking between-patient variations into consideration, averaged AD and E2 production was not substantially different in follicles <10 mm harvested in the 3 groups. This may suggest that normal mechanisms of early folliculogenesis take place in polycystic ovaries. Pooling itself, and the inclusion of large cystic forms of follicles in follicular fluid could explain, next to assay methods improvement, these disparate findings. Since cultured granulosa cells obtained from polycystic ovaries failed to produce significant amounts of E2 when supplemented with AD without FSH - E2 being generated when FSH was added to the milieu -, absence of FSH-induced aromatization was put forward as a pathogenic factor in PCOS women (Erickson et al. 1979). In the present study, however, the proportion of healthy follicles did not vary significantly between the 3 groups. This indicates that normal aromatase activity may be present in follicles of polycystic ovaries. Although E2 production was observed in polycystic ovaries, dominant follicles failed to appear in PCOS and TSX women, suggesting that local mechanisms enhancing aromatization may be disturbed.

As compared to controls, hyperandrogenemia was a common finding in PCOS and TSX subjects with polycystic ovaries, whereas median AD levels were not different in follicles in all three groups. This may indicate, in keeping with previous in vitro work (Wilson et al. 1979), and morphological studies (Haney et al. 1986), that hyperandrogenemia could be due to an abnormally high number of atretic follicles, and not to an augmented androgen production per follicle unit. Although the administration of large amounts of androgens to TSX patients did not seem to affect initiation of

folliculogenesis, follicular growth above the size of 10 mm did not take place. Since INH may modulate androgen biosynthesis in the human (Hillier et al. 1991), we investigated whether INH immunoactivity would be higher in polycystic as compared to normal ovaries. INH bioactivity was found to be raised in follicles < 8 mm harvested from polycystic ovaries (Tanabe et al. 1983), but our findings do not support such a conclusion. In agreement with Buckler et al. (1988) who measured immunoactive INH in serum, no evidence of a defect in ovarian INH production in women with polycystic ovaries could be established.

In conclusion, our data do not sustain the absolute hypo-estrogenic state within polycystic ovaries reported so far in literature (Short & London 1961, Giorgi 1963, Mahesh & Greenblatt 1962, Axelrod & Goldzieher 1962, Jeffcoate et al. 1968). They are nevertheless in keeping with our observations that PCOS women generally have normal early and mid-follicular phase E2 serum levels (Fauser et al. 1991b), which may hardly be explained by the sole peripheral aromatization. In addition, given that the proportion of healthy follicles was not different in normal and in polycystic ovaries, a paradoxical situation could be faced, in that polycystic ovaries may contain larger absolute numbers of healthy follicles than in normal ovaries because of their abnormally large volumes. Altogether, observations in the present study combined with previous reports (Goldzieher 1981, Erickson et al. 1990, Pache et al. 1990, Pache et al. 1991b), may suggest that perturbation of the mechanisms of selection are crucial in the pathogenesis of polycystic ovaries. Since immuno- and bioactive serum levels of FSH are normal in PCOS (Fauser et al. 1991b), disturbed FSH action at the follicular level could be present. The exact role androgens may play in this regard remains to be determined.

Summary

Objective: the aim was to carefully monitor follicular growth arrest in polycystic ovaries by appraising hormone estimates in individual follicles.

Design and patients: fluid of follicles ≤ 10 mm was obtained from ovaries of 16 regularly cycling women between cycle day 1 and 12 of the follicular phase (controls, n=120 follicles), polycystic ovaries of 5 women affected by the polycystic ovary syndrome (PCOS, n=43), and polycystic ovaries from 14 long term testosterone (T) treated female to male transsexuals (TSX, n=120).

Measurements: fluid was assayed for oestradiol (E2), androstenedione (AD), and immunoactive inhibin (INH). Luteinizing hormone (LH), follicle-stimulating hormone (FSH), and T levels were estimated in serum.

Results: median serum LH was lower in TSX than in controls ($P<0.05$), and in PCOS ($P<0.01$). Median serum T was not significantly different between PCOS and TSX, and was elevated in both groups as compared to controls ($P<0.01$). E2 was present in all follicles obtained from polycystic ovaries of PCOS and TSX, in which no follicle >10 mm could be detected. In the 3 groups, between-patient differences in mean E2, AD, INH, and AD/E2 were significantly larger than expected in view of the

variation between follicles within individuals. Taking into account this between-patient difference, no significant differences could be established between controls, PCOS, and TSX for all endocrine parameters. The percentage of presumed healthy follicles (AD/E2 ratio $\leq$4) was 12% in controls, 17% in PCOS, and 14% in TSX, and was not significantly different between groups.

Conclusions: the results presented in this study may indicate that: 1) abnormally high circulating androgen concentrations with or without elevated LH levels disturb the process of selection, and could therefore play a pivotal role in the pathogenesis of polycystic ovaries, 2) in polycystic ovaries from PCOS and TSX, aromatase activity is present in vivo in small antral follicles, and the proportion of presumed healthy follicles is not different from that encountered in normal ovaries, 3) E2 levels are not different between non-dominant follicles of normal and polycystic ovaries, suggesting that only enhancement of aromatase activity by FSH may be disrupted in polycystic ovaries, 4) because AD levels are not different comparing follicles of normal and polycystic ovaries, hyperandrogenemia in PCOS seems to originate from the abnormally high number of cystic atretic follicles generally observed in polycystic ovaries. 5) marked variation in the endocrine follicular micro-environment within- and between-women precludes pooling fluid from several follicles.

Chapter 8

Conclusions

In the classical concept of endocrine regulation of follicular maturation, LH-dependent theca cell androgen production and FSH-induced granulosa cell aromatase activity play a major role. However, gonadotropin and steroid estimates in peripheral blood cannot explain all clinical conditions, and evidence is accumulating that intra-ovarian modulation of gonadotropin action is crucial for regulation of gonadal function. Monitoring of follicle maturation at the ovarian level is therefore mandatory. In this context, the size of a follicle can be evaluated by sonography, which provides information on its developmental stage.

In this thesis, normal and abnormal ovarian follicle growth was assessed by correlating high frequency transvaginal sonography findings with peripheral blood and follicular fluid estimates. A transvaginal sonography scanning method was validated to obtain in vivo accurate and reproducible data on human follicle growth. Reference values were obtained on normal development of non-dominant follicles. This provided a sound basis to explore follicular growth under abnormal conditions, and it appears from the present work that follicular growth arrest takes place in polycystic ovaries, presumably linked to disrupted selection. In this regard, special note should be made of the observation that immuno- and bio-FSH appear to be normal in peripheral blood of PCOS women. Since failure of FSH-induced aromatization was demonstrated in polycystic ovaries, this may indicate disrupted FSH function at the granulosa cell level. Preliminary studies are under way to estimate whether FSH receptor binding may be involved. Besides, the study of follicular fluid in polycystic ovaries performed in the present thesis provides provocative findings in that E2 levels in follicles up to 8 mm are much higher than previously published, and not different from E2 content in similarly sized follicles of normal ovaries. The same is true for INH and AD. This could be an additional strong point in favor of normal early follicular growth followed by disturbed selection in polycystic ovaries. This concept should be further challenged in vitro in human granulosa cells.

Since hyperandrogenemia is a classical feature in PCOS women, consideration was given to the possibility that androgens could play an intermediary role in follicle growth arrest. This presumption was supported by our observation that abnormally high androgens alone induce characteristical polycystic changes in human ovaries. On the other hand, it was found that AD levels within the follicle microenvironment are within normal limits. This indicates that hyperandrogenaemia of PCOS may be linked chiefly to the abnormally high number of cystic and atretic follicles generally observed in polycystic ovaries. Studies attempting to elucidate how exceedingly high androgens may operate at the theca-granulosa level will doubtless broaden our insight into the mechanisms of these changes. In this context, the transsexual model appears to be appropriate, and androgen receptor studies using immunocytochemistry have already been initiated. IGFs and their binding proteins (IGF-BPs) have been implicated as

potential players of follicular development regulation. At least six different IGF-BPs have been identified to date, and Western ligand blod analysis estimating these proteins in follicular fluid from normal and polycystic ovaries are under way. In this regard, Cataldo & Guidice (1992) very recently detected elevated IGF-BPs 2 and 4 in follicular fluid of PCOS patients, and suggested that these IGF-BPs could bind IGF, thereby inhibiting IGF action on the granulosa during normal folliculogenesis.

Concerning the pathogenesis of polycystic ovaries, no less relevant is the emerging role of various LH isoforms in peripheral blood. To what extent gonadal steroids may modulate gonadotropin synthesis is controversial, and the significance of finding elevated levels of bioactive LH in PCOS women remains obscure. The other way around, it could be suspected that LH isohormones with different half-lives and biological activities will have a different effect at the theca cell level, and thereby play an important modulatory role of follicular development.

For clinical purposes, two observations from the present work may be of interest. Firstly, normal FSH in peripheral blood of PCOS women in concert with an observed LH/FSH ratio below 2 by using IRMA methods, underscores the necessity of adapting the LH/FSH reference value to its own laboratory. Also, one could question the utility of a perennial use of this ratio as a diagnostic standard of PCOS. Secondly, criteria to diagnose polycystic ovaries by transvaginal sonography are proposed. They may serve in infertility diagnosis and treatment, and are presently used to ground a reappraisal of the incidence of polycystic ovaries in the general population (Pache et al., unpublished data).

Much remains to be done before intimate intra-ovarian mechanisms can be elucidated in polycystic ovaries. Based on the concept of disturbed selection in polycystic ovaries, Schoot and colleagues (1992b) already demonstrated that monofollicular growth can be obtained with decreasing doses of human menopausal gonadotropin in PCOS women, after selection has been induced. This also emphasizes that local dysregulation of FSH action is a relative defect which can be overcome by elevating FSH with the administration of gonadotropins. Based on the present work, in vitro studies with human granulosa cells were initiated to investigate local regulatory mechanisms (Schipper et al. 1992), and molecular biology investigations are on the verge of being processed. Hopefully, this will broaden our insight into the mechanisms of disturbed follicular growth in PCOS, described by Adashi (1991) as one of the major residual challenges of reproductive endocrinology.

Chapter 9

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Summary

Chapter 1

Some aspects of the current knowledge of ovarian function and dysfunction are reviewed as an introduction to the objectives of the present thesis. It is proposed that in vivo observation - via high frequency transvaginal examination (TVS) and analysis of follicular fluid content -of the ovaries correlated to clinical and peripheral endocrine findings may be an optimal way to gain insight into normal menstrual cycle physiology, and the polycystic ovary syndrome (PCOS) pathophysiology.

Chapter 2

Detailed histological aspect and steroidogenic functions of the ovary under physiological conditions are presented. Based on morphological studies, it is believed that follicular maturation extends over an 85 days period. Today concept of follicular development - initiation of folliculogenesis, recruitment and selection - is highlighted.

Chapter 3

Former echoscopic reports on ovarian follicular development are discussed. Accuracy and reproducibility of TVS to picture follicles as small as 2 mm in diameter are established. Growth of non-dominant follicles - between 2 and 10 mm - in normally cycling women is characterized by using TVS. It is observed that a dominant follicle is selected on average around the seventh day of the menstrual cycle, at a diameter below 10 mm. Besides, non-dominant follicles diameter always remains < 11 mm. Altogether, these observations have implications for the sonographic diagnosis of anovulation and monitoring of ovulation induction.

Chapter 4

The basic concepts of PCOS are surveyed. In depth description of polycystic ovaries histology is provided, and prevailing conjectures about PCOS pathogenesis are scrutinized. Difficulties in diagnosis and treatment of PCOS may arise from observational discrepancies in the largest series: clinical signs in 70% together with sonographic parameters of polycystic ovaries in 30 to 70%, and endocrine signs in 40% only of PCOS patients.

Chapter 5

Previous efforts to find an animal model for the study of PCOS are evaluated. A human model to study the effects of androgens on ovarian tissue is established in female to male transsexuals (TSX). Findings meet the histological criteria for the

diagnosis of polycystic ovaries, and demonstrate that androgens alone may induce polycystic changes. The assumption that the role of androgens is crucial at the follicular level in the genesis of polycystic ovaries is reinforced.

Chapter 6

Growth of ovarian follicles is assessed, firstly in women with irregular cycles and infertility, secondly in PCOS women. The usefulness of TVS in demonstrating correlations between ovarian structure and clinical or endocrine parameters in menstrual cycle disturbances is validated, and criteria to diagnose polycystic ovaries by TVS are subsequently proposed in a controlled study. Most normal ovaries are not larger than 8.0 ml, do not contain more than 11 follicles sized 2 to 10 mm, and do not exhibit an increased stroma echogenicity. These values are highly specific, but sensitivity is poor. The greatest power of discrimination between normal and polycystic ovaries is obtained by combined measurement of follicle size and ovarian volume.

Chapter 7

Steroid and gonadotropin levels are assessed in women with cycle abnormalities only, in patients with PCOS, and compared to measurements obtained from normally cycling women. Gonadotropins are measured by RIAs, IRMAs, and in vitro BIOs. Since previous work has established that stimulation of FSH-induced aromatase activity is disrupted in polycystic ovaries, the concept of follicle maturation arrest in polycystic ovaries is worked out. It is shown that IRMA-FSH, RIA-FSH, and BIO-FSH are not different in all above mentioned 3 groups, and intra-ovarian dysfunction of FSH function is suspected. It is also demonstrated that the LH/FSH ratio >3 - classically used to diagnose PCOS - may need to be revised for IRMA.

The significance of single serum LH estimates by IRMA and BIO for PCOS diagnosis is further evaluated in women with cycle abnormalities, together with the androgen modulation of LH biopotency. Observations underline the heterogeneity of clinical and endocrine characteristics of PCOS, and it is shown that BIO-LH levels do better than IRMA-LH in predicting PCOS. This finding points to the possible importance of various LH isoforms in PCOS pathogenesis. Relationship between immuno- and BIO-LH levels and ovarian structural changes in women with irregular cycles is evaluated in a subsequent study. BIO-LH correlates slightly better than I-LH with echoscopic (TVS) parameters of polycystic ovaries, such as elevated follicle number, ovarian volume and stroma amount. However, the effect of T on ovarian structure is found to be independent from I-LH and BIO-LH. In addition, T levels alone are statistically significant predictors of ovarian changes, with no additional predictive value gained from parallel I-LH or BIO-LH estimates. As a consequence, the independent effect of androgens on polycystic ovarian changes is highlighted, further substantiating their apparent cardinal role in the genesis of polycystic ovaries. In the same study, ovarian changes estimated by TVS reflect coincidental alterations

of clinical and endocrine findings associated to PCOS, showing that TVS assessment of the ovaries can be a valuable additional tool for the diagnosis of the syndrome.

In a fourth study, hormone estimates are performed in individual follicles of normal and polycystic ovaries. No difference in estradiol, androstenedione, and inhibin levels can be found between non-dominant follicles in normal and polycystic ovaries. That exceedingly high circulating androgen concentrations with or without elevated LH levels can disturb follicle selection is documented. Disrupted FSH-enhancement of aromatase activity in polycystic ovaries is hypothesized.

Sammenvatting

Hoofdstuk 1

Enige aspecten van de huidige kennis omtrent ovariumfunctie onder normale en pathologische condities worden belicht als introductie van de doelstellingen van dit proefschrift. Het concept woordt naar voren gebracht dat observatie van ovariumfunctie in vivo - met behulp van transvaginale echoscopie (TVE) en follikelvocht analyse - en mogelijke correlaties met klinische en perifeer endocriene bevindingen een alternatieve manier kan zijn om meer inzicht te krijgen in de fysiologie van de normale menstruele cyclus en in de pathofysiologie van het polycysteus ovarium syndroom (PCOS).

Hoofdstuk 2

Histologische aspecten van het ovarium en steroid produktie door het ovarium onder normale omstandigheden worden in detail belicht. Huidige inzichten omtrent follikelgroei, initiëring van folliculogenese, recruitment en selectie van de dominante follikel worden weergegeven.

Hoofdstuk 3

Echoscopische publicaties omtrent follikelgroei worden bediscussieerd. Nauwkeurigheid en reproduceerbaarheid van TVE om follikels vanaf 2mm in diameter waar te nemen wordt vastgelegd. Groei van de niet dominante follikels - tussen 2 en 10 mm - bij vrouwen met een normale regelmatige cyclus wordt gekarakteriseerd. Waargenomen werd dat een dominante follikel wordt geselecteerd rond de 7e cyclusdag, bij een diameter van 10 mm. De diameter van niet dominante follikels blijft altijd beneden de 11 mm. Deze bevindingen lijken van belang voor de echoscopische diagnose bij anovulatie en voor monitoring van inductie van ovulatie.

Hoofdstuk 4

Het basis concept van PCOS wordt besproken. De histologie van polycysteuze ovaria alsmede de pathogenese van PCOS staan hierbij centraal. Problemen bij diagnose en behandeling van PCOS kunnen voorkomen uit waargenomen discrepanties bij grote patienten groepen met PCOS: in 70% een combinatie met klinische kenmerken, in 30-70% combinatie met polycysteuze ovaria bij echoscopisch onderzoek, en in 40% een combinatie met endocriene stigmata.

Hoofdstuk 5

Pogingen om een diermodel te vinden voor de studie van PCOS worden besproken. Een humaan model om de effecten van androgenen op het ovarium te

bestudeeren zijn vrouw-man transsexuelen. Ovaria van deze patienten vertonen histologisch sterke overeenkomsten met polycysteuze ovaria, en onderstrepen het belang van androgenen voor het ontstaan van PCOS.

Hoofdstuk 6

Een schatting werd gemaakt van de groei van ovariële follikels bij vrouwen met irregulaire cycli en infertiliteit en bij vrouwen met PCOS. De toepassing van TVE werd gevalideerd door bij vrouwen met cyclusafwijkingen te kijken naar correlaties tussen ovariële veranderingen en klinische en endocriene criteria. In een gecontroleerde studie werd vervolgens gezocht naar criteria om polycysteuze ovaria met behulp van TVE te diagnostiseren. In het algemeen zijn normale ovaria niet groter dan 8.0 ml, bevatten niet meer dan 11 follikels met een doorsnede van 2 to 10 mm, en hebben een normale hoeveelheid stroma. Deze criteria zijn zeer specifiek, echter de sensitiviteit is laag. De grootste kans om een onderscheid te maken tussen normale en polycysteuze ovaria wordt verkregen door een gecombineerde meting van follikelgrootte en ovarium volume.

Hoofdstuk 7

Een schatting van steroid en gonadotrofine spiegels werd gemaakt bij vrouwen met cyclusafwijkingen, bij vrouwen met PCOS en bij vrouwen met een regelmatige cyclus. Gonadotrofine bepalingen werden verricht met behulp van RIA's, IRMA's en in vitro bioassays. IRMA-FSH, RIA-FSH en BIO-FSH spiegels zijn niet verschillend in de drie boven genoemde groepen. Op basis daarvan mag en gestoorde intraovariële FSH werking worden verondersteld. Eveneens werd aangetoond dat een LH/FSH ratio boven 3 - veelal gebruikt voor PCOS diagnose - revisie behoeft indien IRMA wordt toegepast. De betekenis van een enkelvoudige LH bepaling met behulp van IRMA or BIO voor PCOS diagnose werd geëvalueerd bij vrouwen met cyclusstoornissen. Verkregen gegevens onderstrepen de heterogeniciteit van PCOS, waarbij tevens duidelijk werd dat BIO-LH spiegels PCOS beter voorspellen dan IRMA-LH. Verschillen in LH isovormen spelen mogelijk een rol in de pathogenese van PCOS. In een vervolg studie werd gekeken naar de relatie tussen IRMA- en BIO-LH spiegels en ovariële veranderingen zoals waargenomen met TVE bij vrouwen met cyclusstoornissen. BIO-LH correleert iets beter dans IRMA-LH met echoscopische parameters van polycysteuze ovaria. TVE kan een waardevolle aanvuling zijn voor de diagnose PCOS.

In een vierde studie werden hormoonspiegels bepaald in individuele follikels van normale en polycysteuze ovaria. Verschillen voor oestradiol, androstenedione of inhibine konden niet worden aangetoond tussen niet dominante follikels afkomstig uit normale of polycysteuze ovaria. Hoge androgeen spiegels al dan niet in combinatie met verhoogde LH spiegels kunnen follikelselectie verstoren. En gestoorde stimulering van aromatase activiteit door FSH zou hieraan ten grondslag kunnen liggen.

Hoofdstuk 8

In de conclusie sectie worden relevante bevindingen verkregen uit dit proefschrift kort bediscussiëerd.

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List of publications included in the present thesis (numbers refer to chapters)

[3]TD Pache, JW Wladimiroff, FH de Jong, WC Hop, BCJM Fauser. Growth patterns of non-dominant ovarian follicles during the normal menstrual cycle. Fertility and Sterility 1990;**54**:638-42

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[7]TD Pache, WCJ Hop, FH de Jong, RA Leerentveld, H v Geldorp, TMM van de Kamp, LJG Gooren, BCJM Fauser. 17-ß estradiol, androstenedione, and inhibin levels in fluid from individual follicles of normal and polycystic ovaries, and in ovaries from androgen treated female to male transsexuals. Clinical Endocrinology (Oxf) 1992;**36**:565-71

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TD Pache, JW Wladimiroff, BCJM Fauser. In vivo evidence for the existence of paracrine regulatory mechanisms in the ovary. VII World Congress of Human Reproduction (Helsinki, Finland), 1990, Abstract 123

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Curriculum Vitae auctoris

Thierry Daniel Pache was born on the 5th of June 1956 in Lausanne, Switzerland. From 1967 to 1976, he studied at the Collège Secondaire de Villamont and Gymnase du Belvédère in Lausanne, and obtained his baccalauréat ès Lettres in the year 1976. Thereafter, he studied medicine at the Faculté de Médecine de l'Université de Lausanne, and got his M.D. diploma in January 1984. Pre-graduate practice took place at the Universities of Montréal (Canada), Costa-Rica (Central America), New York State at Buffafo (USA), and Harvard Medical School (USA). In the years 1984-1986 he was a general practionner for a short time in the mountains (Les Diablerets, Switzerland), resident in neurosurgery and E.N.T. surgery at the Centre Hospitalier Universitaire Vaudois (CHUV), Lausanne, and resident in general surgery at the Hôpital de Morges, Morges, Switzerland. Thereafter, he entered his residency in obstetrics & gynaecology at the Hôpital de Morges, where he stayed up to 1989. In 1988, he obtained the Certificat Universitaire en échographie gynécologique et obstétricale at the University of Strasbourg, France. In 1988-1989, he wrote a M.D. thesis in the field of obstetrical ultrasound and obtained a Doctorate in Medicine. From april 1989 up to august 1991, he worked at the Department of Obstetrics & Gynaecology of the Dijkzigt Academisch Ziekenhuis, Rotterdam, The Netherlands, Section of Reproductive Endocrinology and Infertility. Since the 1st of october 1991, he has been at the Department of Obstetrics & Gynaecology of the CHUV, Lausanne, Switzerland.

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