AGE-RELATED ANDROGEN SECRETION IN HEALTHY WOMEN AND IN WOMEN WITH POLYCYSTIC OVARY SYNDROME

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Abstract

The number of ovarian follicles declines with age resulting in a significant decrease of fertility by the age of 40. However, the age when follicle loss starts to affect ovarian endocrine function is not well recognized. The purpose of the present study was to investigate age-related ovarian/adrenal androgen secretion, which is crucial for estrogen biosynthesis in healthy women and in women with polycystic ovarian syndrome (PCOS). Another aim of the study was to compare the usefulness of different serum markers in assessing ovarian aging and in diagnosing polycystic ovaries (PCOs) and PCOS.

The human chorionic gonadotropin (hCG) test was used to study the endocrine potential of ovaries/adrenals. The ovarian capacity to secrete and synthesize androgens was found to be decreased as early as at the age of 30 in regularly menstruating women. In women with PCOS, both basal and hCG-stimulated androgen levels were about 50% higher than in healthy women and they remained high until late reproductive age. Similarly to regularly menstruating women, the androgen secretion capacity in PCOS subjects decreased with age, and estradiol concentrations remained unchanged until the age of 44 years. Adrenal androgen synthesis was not changed during hCG-tests. Since serum antimüllerian hormone (AMH) and follicle stimulating hormone (FSH) levels were changed significantly after the age of 25 years in regularly menstruating women, they may be considered as useful serum markers reflecting the ovarian aging process. In women with PCOS, AMH levels were continuously 2- to 3-fold higher than in healthy women possibly reflecting high follicle number in these women.

A decline in ovarian endocrine function before the age of 30 is one of the first signs of ovarian aging. However, in women with PCOS ovarian androgen secretion capacity is markedly increased and remain high throughout the reproductive years. The results of the present studies also indicate that LH/hCG does not play a role in adrenal androgen synthesis, since LH/hCG did not stimulate adrenal androgen synthesis. The measurement of AMH is a useful tool to estimate ovarian aging process as well as to diagnose PCOs/PCOS.

Keywords: androgen secretion, antimüllerian hormone, hCG-test, ovarian aging, polycystic ovary syndrome
"A candle loses nothing of its light by lighting another candle."

- James Keller

To my Family
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Abbreviations

A  androstenedione
ACTH  adrenocorticotropic hormone
AMH  antimüllerian hormone
AUC  area under the curve
BMI  body mass index
CC  clomiphene citrate
CPA  cyproterone acetate
CRH  corticotrophin-releasing hormone
DHEA  dehydroepiandrosterone
DHEAS  dehydroepiandrosterone sulphate
DHT  5α-dihydrotestosterone
E2  oestradiol
ERT  oestrogen replacement therapy
FFA  free fatty acid
FOH  functional ovarian hyperandrogenism
FSH  follicle-stimulating hormone
GnRH  gonadotrophin-releasing hormone
GnRH-a  gonadotrophin-releasing hormone agonist
hCG  human chorionic gonadotrophin
3β-HSD  3β-hydroxysteroid dehydrogenase
17β-HSD  17β-hydroxysteroid dehydrogenase
IGF  insulin-like growth factor
IGFBP  insulin-like growth factor-binding protein
IGT  impaired glucose tolerance
IR  insulin resistance
i.m.  intramuscular
17-OHP  17-hydroxy progesterone
LH  luteinizing hormone
MIF  müllerian inhibiting factor
MIS  müllerian inhibiting substance
NCAH  non-classic adrenal hyperplasia
OC  oral contraceptive
P  progesterone
P450scc  cholesterol side chain cleavage enzyme
PA  premature adrenarche
PCO  polycystic ovary
PCOS  polycystic ovary syndrome
POF  premature ovarian failure
PRL  prolactin
RIA  radioimmunoassay
SHBG  sex hormone-binding globulin
SK  sulphokinase
SL  sulpholyase
T  testosterone
TGF  transforming growth factor
WHR  waist/hip ratio
List of original articles

This thesis is based on the following articles, which are referred in the text by their Roman numerals:


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References
1 Introduction

In the human ovary, the majority of the 1–2 million follicles present at birth become atretic by undergoing apoptosis, and only about 400 of them will eventually ovulate during reproductive life (Gougeon 1996). The period of optimal fertility lasts until the age of about 30 and decreases gradually thereafter (van Noord-Zaadstra et al. 1991, te Velde et al. 1998b, Wu et al. 2000). The follicle pool is decreased significantly by the age of 37–38 with concomitant increases in infertility and the miscarriage rate (Meden-Vrtovec 2004). Menopause occurs around the age of 50 years, when the number of follicles is reduced to almost zero and menstrual cycles stop. Follicles, together with stromal tissue, are responsible for ovarian steroid production and oestrogen biosynthesis. Even though many investigators have studied age-related oestrogen synthesis, especially in perimenopausal and postmenopausal women (Rozenberg et al. 1988, Burger et al. 1999), the age-related changes in androgen secretion are poorly understood. There are several factors, such as FSH, inhibin B and AMH that have been used as markers of ovarian function/aging but the data concerning their clinical usefulness are conflicting. Besides ovarian androgen synthesis, the adrenals also contribute to total androgen production, and with aging there is a marked continuous decline in adrenal androgen levels (Orentreich et al. 1984).

Androgen secretion is necessary for estrogen biosynthesis. The important physiological role of androgens is best seen in women who suffer from androgen insufficiency (Female Androgen Insufficiency Syndrome). These women suffer from decreased well-being, blunted motivation, persistent unexplained fatigue, sexual dysfunction including lowered libido, sexual receptivity or pleasure, decreased bone mineral density, decreased muscle strength and changes in cognition and memory (Bachmann et al. 2002). Similarly, hyperandrogenism causes many unfavourable changes in female physiology. The most common cause of hyperandrogenism in women of fertile age is PCOS. Such women suffer from menstrual irregularity, infertility, obesity, hirsutism, acne and metabolic disorders such as type-2 diabetes. Other reasons for hyperandrogenism include ovarian or adrenal enzyme defects, adrenal hyperplasia, hyperprolactinaemia and tumours.

In the present study, the age-related changes in ovarian androgen synthesis/secretion were studied in healthy (regularly cycling) women and in women with PCOS, using basal
and hCG (an analogue of LH) -stimulated hormone measurements. In addition, different serum markers that have been thought to reflect ovarian follicle and hormonal reserves were investigated and compared. The role of the adrenals in female androgen secretion was studied by means of hCG tests in oophorectomized postmenopausal women and in regularly cycling women during ovarian suppression by means of GnRH agonist treatment.
2 Review of the literature

2.1 Androgen secretion in women

In women, androgens and androgen precursors are secreted by the ovaries and adrenal glands in response to their trophic hormones LH and adrenocorticotropic hormone (ACTH). Some androgens are also produced indirectly through peripheral metabolism of secreted precursors. In the ovaries androgens are mainly produced in theca cells and in the adrenal glands in the cortex. It has been estimated that 25% of T production is ovarian in origin, 25% is adrenal in origin and 50% is derived from A in peripheral tissues (Longcope 1986). A is also produced equally (25%-50%) from ovaries and adrenals, and the rest is produced through peripheral metabolism, whereas DHEAS is almost 100% of adrenal origin (Orentreich et al. 1984, Longcope 1986, Morley & Perry 2003).

The first rate-limiting step in the formation of all steroids both in ovaries and in adrenal glands is the conversion of cholesterol to pregnenolone which is regulated by pituitary trophic hormones (LH and ACTH) and carried out through regulation of gene expression of the cholesterol side chain cleavage enzyme P450ssc (Rosenfield 1999). Another important step in androgen synthesis is P450c17 action (17-hydroxylase and 17,20-lyase activity), required for DHEA and A production from pregnenolone and progesterone. Other important enzymes include 3\(\beta\)-hydroxy steroid dehydrogenase (3\(\beta\)-HSD), which catalyzes conversion of pregnenolone to progesterone and DHEA to A, and 17\(\beta\)-hydroxy steroid dehydrogenase (17\(\beta\)-HSD), which catalyzes the conversion of A to T (Fig 1).

In the circulation androgens are bound to protein carriers such as sex hormone-binding globulin (SHBG) and albumin, leaving only few percent of androgens unbound and free (Longcope 1998, Vermeulen 1998). The biological effects of androgens are largely determined by the unbound portion. Several factors that influence SHBG concentration also affect androgen levels (Pugeat et al. 1981). Oral oestrogen and thyroxine are known to increase SHBG levels, whereas obesity, growth hormone, hyperinsulinaemia, insulin-like growth factors (IGFs) and glucocorticoids all decrease serum SHBG levels (Kalme et al. 1999, Kaltsas et al. 2000, Pugeat et al. 2000, Hampl et al. 2003, Kalme et al. 2003).
Since A, DHEA and DHEAS are considered as pro-androgens they require conversion to T and dihydrotestosterone (DHT) in the target cell to express their androgenic effects. According to the current concept of androgen action free T enters the cell through the plasma membrane by either passive or facilitated diffusion. The cytoplasm of many target cells contains the enzyme 5α-reductase, which converts most of the T to DHT. When T/DHT binds to nuclear receptor it causes activation of specific genes that code certain proteins. These protein products mediate many, if not all, of the effects of androgens.

2.1.1 Ovaries

Gonadotrophin-releasing hormone (GnRH), which is secreted from the hypothalamus in a pulsatile fashion, regulates pituitary LH and FSH secretion through the portal vasculature. Luteinizing hormone and FSH control ovarian androgen and oestrogen biosynthesis. According to the two-cell-two-gonadotrophin model of ovarian function (Fig. 1), since androgens are produced in the theca cells in response to pituitary LH secretion and then diffused to the granulosa cells where they are converted to oestrogens by aromatase enzyme activity under the influence of FSH. Oestrogens in turn alter both hypothalamic and pituitary secretory patterns, constituting a regulatory loop system between brain and ovary.

Fig. 1. Ovarian steroid production in the small antral follicles according to the two-cell-two-gonadotrophin model. GnRH is produced in the hypothalamus, regulating pituitary gonadotrophin (LH, FSH) release. LH stimulates androgen formation in the theca cells, whereas FSH stimulates aromatase activity in the granulosa cells promoting the metabolism of androgens to oestrogens.
Even though androgen production is necessary for oestrogen biosynthesis, an excess of androgens might disturb both follicular growth and development. To ensure an optimal environment for follicle function, androgen synthesis is strictly regulated during the whole of the menstrual cycle. At the beginning of the menstrual cycle steroid production is at its lowest and it increases towards ovulation (Apter et al. 1978)(Fig. 2). Close to midcycle there is positive oestrogen feedback on the pituitary/hypothalamus and a concomitant loss in the sensitivity to negative feedback inhibition, causing increase in both in LH and FSH secretion just before ovulation. At this point the androgen levels are also at their highest (Apter et al. 1978). Another high peak in androgen levels occurs in the luteal phase. If the ovulated oocyte is not fertilized, androgen levels decrease, being again at their lowest at the beginning of the next cycle (Vermeulen & Verdonck 1976, Apter et al. 1978, Vihko & Apter 1980, Wajchenberg et al. 1989).

Fig. 2. Changes in serum hormone levels during the menstrual cycle in healthy regularly menstruating women.
Regulation of intraovarian androgen concentrations is necessary for normal ovarian function. Androgens are substrates for oestrogen (mainly estradiol [E2]) biosynthesis and promote the growth of small follicles (Hillier & Tetsuka 1997, Vendola et al. 1998) but an excess interferes with follicular maturation, preventing the emergence of the dominant follicle and committing the follicle to atresia (Hillier & Tetsuka 1997). Although E2 has an efficient feedback mechanism, androgens, serving as byproducts of E2 synthesis, do not have a straight negative feedback system, exposing women to uncontrolled hyperandrogenism (Serafini et al. 1986, Ehrmann et al. 1995).

2.1.2 Adrenals

Adrenal androgen secretion starts when the adrenals go through “adrenal puberty” known as adrenarche. In this adrenal maturation the adrenal cortex develops the ability to secrete 17-ketosteroids and there is also a change in the pattern of the adrenal response to ACTH. The new secretory pattern is characterized by increase in 17-hydroxypregnenolone and DHEA responsiveness to ACTH, whereas cortisol responsiveness remains unchanged (Rich et al. 1981). Although adrenarche is unrelated to gonadal puberty, ovarian function may have a role in supporting adrenal DHEAS production since oophorectomy causes an early decline in DHEAS levels (Cumming et al. 1982). Moreover, it is unlikely that ACTH exclusively regulates adrenal androgen production since several investigators have reported dissociation between cortisol production and that of adrenal androgens (McKenna & Cunningham 1991, Parker 1991b, McKenna et al. 1997). The presence of receptors for hCG (Pabon et al. 1996, Mircescu et al. 2000, Lacroix et al. 2001), insulin, insulin-like growth factor 1 (IGF-1) (Penhoat et al. 1988, Pillion et al. 1989) and prolactin (PRL) (Glasow et al. 1998) in the adrenal cortex raises the possibility of their involvement in the control of adrenal steroidogenesis.

The adrenal cortex produces three groups of steroid hormones, the glucocorticoids, the mineralocorticoids and sex steroids in response to pituitary ACTH secretion. Release of ACTH is regulated by hypothalamic corticotrophin-releasing hormone (CRH). Some of the adrenal sex steroids are used in the synthesis of glucocorticoids and mineralocorticoids (Fig. 3).

Adrenal androgens are synthesized from cholesterol through Δ5 and Δ4 pathways (Fig. 3). P450c17, having both 17-hydroxylase and 17,20-lyase activity, has a central role in ovarian as well as in adrenal androgen production. In contrast to the ovaries, the adrenals use androgens as precursors for aldosterone (mineralocorticoid) and cortisol (glucocorticoid) production. As a result of sulphokinase and sulpholyase enzyme activity in the adrenals, some of the DHEA is transformed into DHEAS. Since DHEAS is almost uniquely derived from the adrenal glands it can be used as marker of adrenal androgen production (Lobo et al. 1981).

Excessive secretion of sex steroids occurs only in neoplastic cells or in association with enzyme deficiencies. Under normal circumstances, the adrenal gland contributes to androgen synthesis equally with the ovaries, although total adrenal sex steroid production is less significant than gonadal production of androgens and oestrogens.
Fig. 3. Adrenal steroid synthesis. Pituitary ACTH regulates adrenal androgen synthesis from cholesterol. The square contains the hormonal cascade that is present both in the ovaries and adrenals. P450c17α, having both 17-hydroxylase and 17α-lyase activities, also has a major role in adrenal androgen production. The adrenals synthesize DHEAS from DHEA by way of sulphokinase (SK) enzyme activity. In addition to androgen secretion, the adrenals also produce aldosterone and cortisol. P450 side chain cleavage (P450scc), sulpholyase (SL).

2.1.3 Peripheral tissues

The major clinically important sites of peripheral androgen conversion are the lung, liver, adipose tissue and skin, as they have both 3β-HSD and 17β-HSD activity. Much (30% to 50%) of the plasma T is derived from the peripheral tissues (Ala-Fossi et al. 1998). Even though T is the major circulating androgen, the principal active androgen in target cells is DHT, which arises almost entirely by way of 5α-reductase activity in the periphery in the target tissue (Silva et al. 1987). Besides the conversion of androgens, peripheral tissues also have aromatase activity, which results in metabolism of androgens to estrogens (Nelson & Bulun 2001).
2.2 Disorders of androgen secretion

2.2.1 Hypoandrogenism

Androgen insufficiency occurs in a number of circumstances, including hypopituitarism, premature ovarian failure, adrenal failure and use of exogenous corticosteroids (Braunstein 2002, Davis & Burger 2003) (Table 1.). Moreover, therapeutic procedures such as bilateral oophorectomy lead to an approximately 50% reduction in androgen production (Judd et al. 1974, Hughes et al. 1991). Patients with Turner’s syndrome and omen with other forms of ovarian dysgenesis may also have low androgen levels (Hojbjerg Gravholt et al. 1999). The clinical symptoms of androgen insufficiency include loss of libido, diminished well-being, fatigue and blunted motivation, decreased bone mineral density, decreased muscle strength and changes in cognition and memory (Bachmann 2002, Ghizzani et al. 2003).

Table 1. Conditions associated with androgen insufficiency and excess in women.

<table>
<thead>
<tr>
<th>Androgen insufficiency</th>
<th>Androgen excess</th>
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<tbody>
<tr>
<td>Hypothalamic-pituitary abnormalities</td>
<td>PCOS</td>
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<tr>
<td>Premature ovarian failure</td>
<td>Premature adrenarche</td>
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<tr>
<td>Adrenal insufficiency</td>
<td>Hyperinsulinaemia</td>
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<tr>
<td>Exogenous corticosteroid therapy</td>
<td>Enzyme defects</td>
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<tr>
<td>Oophorectomy</td>
<td>Adrenal hyperplasia</td>
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<td></td>
<td>Hyperprolactinaemia</td>
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<td></td>
<td>Tumours</td>
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</table>

2.2.2 Hyperandrogenism

Androgen excess is one of the most common endocrine disorders in reproductive-aged women, affecting approximately 7% of this population (Knochenhauer et al. 1998, Asuncion et al. 2000). Androgen excess can result in development of acne, ovulatory dysfunction, hirsutism, androgenic alopecia, virilization and masculinization. Disorders that result in androgen excess include specific disorders such as PCOS, premature adrenarche, non-classic adrenal hyperplasia (NCAH), hyperprolactaemia and androgen-secreting tumours (Table 1).

2.2.2.1 Polycystic ovary syndrome (PCOS)

Polycystic ovary syndrome is the most common reproductive endocrine disorder in fertile women. It was first described in 1935 by Stein and Leventhal (Stein IF 1935) when they reported an association between polycystic ovaries in women with amenorrhoea,
hirsutism and obesity. The heterogeneity of both the ovarian histology and clinical findings in women with polycystic ovaries led to the establishment of the term PCOS. Earlier, the diagnosis of PCOS was based on histological criteria and clinical findings (Goldzieher & Green 1962), but today transvaginal ultrasonography has replaced ovarian biopsy (Dewailly 1997). Even though PCOS is common in women of fertile age, the aetiology is still an open question.

Criteria. Criteria for the diagnosis of PCOS have varied over time. In 1990, at the National Institutes of Health (NIH) Conference held in the USA, three minimal criteria for the diagnosis of PCOS were proposed; 1) menstrual irregularity, 2) evidence of hyperandrogenism and 3) exclusion of other diseases (NCAH, Cushing’s syndrome, hyperprolactinemia and androgen-secreting tumours) (Dunaif et al. 1992). According to these criteria, the morphological diagnosis of polycystic ovaries (PCOs) by ultrasonography was not considered as an essential part of the diagnosis, even though in Europe an ultrasonographic finding of PCOs was one of the main criteria (Homburg et al. 1996). However, as awareness concerning PCOS has increased, the diagnostic criteria and problems related to this syndrome have been reanalyzed.

In the PCOS workshop of the European Society for Human Reproduction (ESHRE) and the American Society of Reproductive Medicine (ASRM), held in Rotterdam in May 2003, the criteria of PCOS were reconsidered and new guidelines were suggested. According to the new criteria a diagnosis of PCOS can be made when two of the following criteria are recognized: oligomenorrhoea and/or anovulation, clinical or biochemical signs of hyperandrogenism and ultrasonographic findings of PCOs (ESHRE/ASRM 2004a, ESHRE/ASRM 2004b).

Epidemiology of PCOS. Data on the prevalence of PCOS are variable, owing in part, to the lack of well accepted criteria for diagnosis, and the heterogeneous phenotype of the syndrome. An ultrasonographic finding of PCOs is seen in 87% of women with oligomenorrhoea (Adams et al. 1986), in 23% of women who report having regular menstruation (Polson et al. 1988) and in about 14% of women of fertile age who have not sought help for their gynecological symptoms (Koivunen et al. 1999). Hirsutism seems to have a strong relationship with PCOS, since 92% of women with idiopathic hirsutism also have PCOs (Adams et al. 1986). If biochemical parameters, such as elevated serum T, A or DHEAS, levels are used as diagnostic criteria, the prevalence of PCOS is estimated to be around 4% (Knochenhauer et al. 1998).

Features of PCOS. In clinical practice, women with PCOS are seen for three major reasons: menstrual irregularity, androgen excess and infertility.

The menstrual irregularity in women with PCOS is chronic, typically beginning at menarche (Yen 1980). Although amenorrhoea may occur, the more typical presentation is irregular bleeding, which is characteristic of anovulation. In polycystic ovaries the growth of antral follicles is arrested at a stage where the follicles are active, producing different hormones, including androgens (Gilling-Smith et al. 1994). Since the follicles do not proceed to the ovulatory stage, the number of small follicles is increased, giving the ovary the typical appearance seen in cases of PCOs (Legro et al. 1998). Besides menstrual/ovulatory disorders, women with PCOS also have infertility problems arising from their anovulatory status.

One of the typical features of PCOS is inappropriate gonadotrophin secretion, seen as high serum LH levels and an increased LH/FSH ratio. Several investigators have
demonstrated an increase in the frequency and amplitude of LH release, with concomitant normal or decreased FSH production (Rebar et al. 1976, Waldstreicher et al. 1988, Taylor et al. 1997). The abnormal LH secretion might reflect an increase in GnRH synthesis, reflecting a possible hypothalamic defect in women with PCOS (Taylor et al. 1997), although other factors may also contribute to this problem (Patel et al. 2004).

The main clinical findings pertaining to hyperandrogenism in women with PCOS are acne and hirsutism. Even though there are several studies concerning androgen synthesis in PCOS, the origin of hyperandrogenism remains an open question. Several studies have demonstrated abnormal theca cell function, causing increased formation of 17-OHP, A and T (Barnes 1989, Gilling-Smith et al. 1994, Nelson et al. 1999, Rosenfield 1999, Strauss et al. 2002). Ovarian stimulation with a GnRH-a or hCG results in higher androgen responses in PCOS subjects than in normal women and speaks for hyperandrogenism of ovarian origin (Barnes 1989, Ehrmann et al. 1992). However, although suppression of the ovaries in women with PCOS with long-acting GnRH antagonists causes a decline in serum androgen levels, the androgen concentrations remain higher than in normal subjects (Chang 1983, Rittmaster & Thompson 1990, Cedars et al. 1992). Supporting the hypothesis that androgen excess in case of PCOS subjects could also be of adrenal origin, some women with PCOS have shown an exaggerated androgen response to ACTH (Anapliotou et al. 1990, Turner et al. 1992). However, despite an enhanced response to adrenal stimulation, suppression of the adrenals with dexamethasone still leaves higher androgen levels in cases of PCOS than in healthy women. Thus, it could be concluded that hyperandrogenism in PCOS is possibly of both ovarian and adrenal origin. According to the results of a recent study, peripheral androgen synthesis may also contribute significantly to hyperandrogenism through increased peripheral 5α-reductase activity observed in women with PCOS (Fassnacht et al. 2003).

In addition to increased theca cell function, some investigators have also reported that women with PCOS have an increased capacity for E2 production in granulosa cells (Mason et al. 1994, Coffler et al. 2003), although the high E2 levels seen in some cases of PCOS may also be derived from peripheral tissues, especially in obese women.

Approximately 30–50% of women with PCOS are obese (Franks 1989, Franks 1995, Conway 1996) usually shown as an increase in abdominal fat. This “male-type obesity”, has been shown to be associated with high lipolytic activity, releasing free fatty acids (FFAs) into the blood circulation (Bouchard & Perusse 1993). Since the FFAs compete with glucose for uptake in muscle and adipose cells, glucose is not used properly, resulting in excessive use of FFAs and a decrease in insulin-mediated glucose utilization (Holte et al. 1994). In the end this leads to impaired glucose tolerance (IGT), insulin resistance and hyperinsulinemia (Nestler 1989, Dunaif et al. 1992, Holte 1996, Morin-Papunen et al. 2000)(Fig. 4). Lean women with PCOS have also been shown to suffer from insulin resistance (Dunaif et al. 1989, Morin-Papunen et al. 2000). Furthermore, according to the results of several studies carried out among women with PCOS, IGT has been related to risks of gestational and type 2 diabetes (Paradisi et al. 1998, Legro 1999, Arslanian 2000, Koivunen et al. 2001, Mikola et al. 2001, Norman 2001, Bjercke et al. 2002). Since hyperinsulinaemia promotes hyperandrogenism, the treatment of women with PCOS with insulin sensitizers such as metformin and troglitazone leads to
improvement in insulin resistance, decreases insulin and androgen levels and improves ovulatory functions (Morin-Papunen et al. 1998, Azziz et al. 2003).

Fig. 4. Metabolic and hormonal disturbances and their relationship in women with PCOS.

Besides the risk of type 2 diabetes mellitus, women with PCOS have also been thought to have an elevated risk of cardiovascular diseases based on the risk factors associated with these conditions (Pierpoint et al. 1998, Loucks et al. 2000, Mather et al. 2000, Talbott et al. 2000). However, since the sample size in these studies have been small and no results from larger prospective epidemiological studies are yet available, the actual link between cardiovascular diseases and risk factors in women with PCOS remains open (Legro 2003).

Obesity and the risk of type 2 diabetes also increase also the risk of endometrial carcinoma in women with PCOS (Dahlgren et al. 1991). The link between endometrial carcinoma and obesity/type 2 diabetes could be elevated oestrogen levels as several investigators have reported an association between high serum E2 levels and endometrial carcinoma. However, the results as regards the risk of ovarian cancer (Schildkraut et al. 1996, Pierpoint et al. 1998) and breast cancer (Balen 2001, Atiomo et al. 2003) in women with PCOS are controversial.

Mechanisms of hyperandrogenism in PCOS. The heterogeneity of PCOS populations has resulted in various hypotheses/studies concerning the mechanism that induces hyperandrogenism in these women. Several investigators have reported excessive androgen production in the theca cells in women with PCOS (Gilling-Smith et al. 1994, Gilling-Smith et al. 1997, Nelson et al. 1999), and there are several factors/mechanisms that have been suggested to be the promoters for this. Disturbances in adrenal androgen production have also been studied.
The results of some studies have suggested that androgen excess is caused by an intrinsic disturbance in the theca cells. Since enzymes P450scc, P450c17, 17β-HSD and 3β-HSD are all required for appropriate androgen synthesis, possible defects in these enzymes have been studied. P450c17 is the only enzyme known to have the capacity to convert C21-precursors to the androgen pre-hormones (17-ketosteroids) by having 17α-hydroxylase and 17,20-lyase activities, and it has a key role in androgen biosynthesis both in the ovaries and adrenals. Some investigators have proposed that increased androgen production in PCOS results from dysregulation of P450c17 (Gilling-Smith et al. 1994). Furthermore, as women with PCOS tend to suffer from insulin resistance and elevated insulin levels, some studies have demonstrated that P450c17 activity is promoted by insulin, and a decrease in insulin levels also reduced androgen levels (Nestler & Jakubowicz 1997, Morin-Papunen et al. 1998). Elevated 17β-HSD activity has also been thought to be associated with increased T levels, although several investigators have reported a normal 17β-HSD activity in the theca cells of PCOS subjects. (Nestler & Jakubowicz 1997, Nelson et al. 1999, Nelson et al. 2001).

Insulin has been shown to promote ovarian androgen secretion in theca cells (Nestler et al. 1998) and to enhance the effect of LH on theca cell function (Barbieri et al. 1986). Insulin acts through its own receptors which are widely distributed in the ovarian tissue (Poretsky et al. 1999), through insulin-like growth factor (IGF) receptors or through insulin/IGF combination receptors (Willis & Franks 1995, Gdansky et al. 1997, Poretsky et al. 1999). High insulin levels also result in decreased serum SHBG levels and in increase of free and biologically active androgens (Nestler 1997, Pugeat et al. 2000). Insulin-like growth factors, previously named somatomedins, are single-chain polypeptides mainly secreted by the liver. They act through their own receptors, affecting cellular mitosis and differentiation in various cell types and regulating ovarian follicular maturation (Adashi et al. 1985, Giordano et al. 1992) and steroidogenesis (Erickson et al. 1990). In the circulation IGFs are mainly bound to specific IGF-binding proteins (IGFBPs), which regulate IGF action through the amount of unbound, free IGF. Insulin decreases IGFBP synthesis in the liver causing an increase in bioactive free IGF levels (Suikkari et al. 1988, Suikkari et al. 1989). The two main IGFs, IGF-I and IGF-II, act through their own receptors and as mentioned above, also insulin can act through these receptors. Alterations in the IGF system, including elevated free IGF-I and decreased IGFBP levels, have been demonstrated in PCOS and may be involved in its pathogenesis (Suikkari et al. 1989, Homburg et al. 1992, Van Dam et al. 2002). For example according to a case report concerning a young woman with PCOS and a concomitant growth hormone-producing pituitary adenoma, the reduction of elevated GH levels to normal by means of dopaminergic agents decreased plasma IGF-I levels and improved ovarian dysfunction (Hashimoto et al. 2003). Menstrual cycles were restored and the number of ovarian cysts was reduced. This would suggest that insulin and/or IGF-I, as stimulators of theca cell proliferation, may be pathogenetic factors in PCOS. Interestingly insulin sensitizers such as metformin and roglitazone have been shown to decrease IGF-I levels and increase those of IGFBP (de Leo et al. 2000, Belli et al. 2004).

Inhibin A and B are ovarian proteins secreted from granulosa cells of growing preantral and antral follicles. They play an endocrine role in co-regulating (with E2) the suppression of FSH during the late follicular and luteal phases of the ovarian cycle and they also have an important role in intraovarian signalling. Even though there is an excess
of small ovarian follicles in women with PCOS, only a few investigators have reported increased inhibin B levels in such women (Anderson et al. 1998, Lockwood et al. 1998, Cortet-Rudelli et al. 2002). Some investigators have observed no difference in inhibin levels between cases of PCOS and healthy women (Magoffin & Jakimiuk 1998, Pigny et al. 2003). Since inhibin B levels have been shown to correlate positively with serum LH levels and inversely with insulin levels and body mass index (BMI), it could be that these factors regulate inhibin B synthesis directly or through some other factors (Welt et al. 1999, Cortet-Rudelli et al. 2002). Whether inhibins play a key role in excessive androgen synthesis in women with PCOS is still open.

Luteinizing hormone has also been suggested to contribute to hyperandrogenism in women with PCOS. Since LH regulates androgen synthesis in theca cells, and since women with PCOS are known to have elevated LH levels, an increased response of theca cells to LH stimulation could be one of the explanations for the hyperandrogenism.

Since circulating androgens are mostly bound to SHBG and albumin the changes in SHBG levels in particular cause alterations in free, bioactive androgen concentrations. Obesity is also known to decrease SHBG levels and the action is probably mediated through insulin since weight reduction increases serum SHBG levels in women with PCOS (Longcope et al. 2000).

Although excessive adrenal androgen production is observed in 40–70% of hyperandrogenic patients (Wild et al. 1983, Knochenhauer et al. 1998), the mechanisms behind it remain open. Extra-adrenal factors such as insulin and E2 concentrations have been shown to correlate with adrenal androgen production (Ditkoff et al. 1995, Martikainen et al. 1996). Since insulin has been shown to stimulate DHEAS production and decrease DHEA synthesis in vitro, it is possible that it has some effect on adrenocortical sulphotransferase activity (Hines et al. 2001). On the other hand, an excess of adrenal androgens might also result from dysfunction of the hypothalamic-pituitary-adrenal axis. However, subjects with PCOS have not been shown to have elevated ACTH levels (Stewart et al. 1993) or an altered pituitary response to CRH (Azziz et al. 1998), which indicates abnormal adrenal function rather than overproduction of the regulating hormones. The fact that some women with PCOS have an increased response to ACTH stimulation (Azziz et al. 1998) supports this hypothesis. An increased response to ACTH stimulation in PCOS might partly result from increased P450c17α activity or factors related to P450c17α function (Rainey et al. 1990, Rosenfield et al. 1990), although other mechanisms might also be involved.

### 2.2.2 Other reasons

One of the causes of hyperandrogenism in women of fertile age could be premature adrenarche (PA). A diagnosis of the premature adrenal maturation is made when there is the appearance of pubic and/or axillary hair before the age of eight years without any other signs of sexual development (Apter et al. 1979). Studies by several investigators have shown that adrenarche is characterized by marked increase in urinary 17-ketosteroid excretion and increased serum levels of T, DHEA and DHEAS (Parker 1991a, Saenger & Dimartino-Nardi 2001), which in girls with PA appears to be associated with
hyperandrogenism and a risk of developing PCOS (Rosenfield 1990, Gonzalez 1997, Dimartino-Nardi 1999). Furthermore, since hyperinsulinaemia, elevated serum IGF-I and decreased IGFBP levels have also been reported in PA, dysregulation of the IGF system may be involved in the pathogenesis of PA and its progression to PCOS (Silfen et al. 2002).

There are several types of 21-hydroxylase defects in adrenal function. Non-classic adrenal hyperplasia has been increasingly recognized in adolescent and adult hyperandrogenic females. These patients have enzyme defects associated with 21-hydroxylase and 11β-hydroxylase deficiency and they also have a higher prevalence of PCOs (Hague et al. 1990). The condition is usually diagnosed at puberty after premature and aberrant growth, with simultaneous acne and amenorrhea.

Antiepileptic drugs, especially valproate have also been shown to result in PCOS and concomitant hyperandrogenism (Isojärvi et al. 1993). Furthermore, some investigators have reported that prolactin affects the Δ5 and Δ4 pathways in the adrenal glands, suggesting that it is able to modulate adrenal androgen production (Lee et al. 1998). In addition, treatments that reduce serum PRL concentrations reduce serum androgen levels (Lobo & Kletzky 1983, Schiebinger et al. 1986, Hagag et al. 2001).

In conditions such as ovarian and adrenal tumours that secrete androgens, there is autonomous, unregulated production and release of androgens independent of the effects of LH/ACTH or other physiological controllers of androgen production. If a tumour secretes high levels of androgens in females, it can lead to virilization, change in voice, clitoromegaly, extreme hair growth, male pattern balding, muscle development and infertility. However, tumors that produce androgens account for only 1% of the cases of hyperandrogenism (Azziz et al. 2004).

### 2.2.2.3 Treatment of hyperandrogenism

The treatment of hyperandrogenism depends on the aetiology of the excessive androgen synthesis as well as on possible related problems/disorders. Hyperandrogenism often causes difficult symptoms such as acne, hirsutism and menstrual irregularities. Commonly, the aim of medical treatment is to decrease ovarian and adrenal androgen production or to block androgen action in the skin.

Oral contraceptives (OCs) have been widely used for the treatment of acne, hirsutism and menstrual irregularities in women with PCOS. They have been shown to reduce ovarian androgen production and to decrease serum androgen levels by up to 50% (Givens et al. 1974, Burkman 1995). In addition OCs have been shown to suppress gonadotrophin secretion (Burkman 1995), inhibit DHT binding to androgen receptor (Eil & Edelson 1984) and increase SHBG synthesis causing a concomitant decrease in free, unbound biologically active androgen levels (Lemay & Poulin 2002). Oral contraceptives also improve irregular menstrual cycles and reduce the risk of endometrial carcinomas (Prelevic et al. 1989, Workshop 2001).

Treatment with GnRH agonists has been shown to improve hirsutism since they affect ovarian androgen production by suppressing pituitary LH secretion (Tiitinen et al. 1994). Hirsutism has also been treated with cyproterone acetate (CPA) and spironolactone, since
they serve as competitive inhibitors of androgens and act through binding to androgen receptors and blocking androgen action on target cells (Kutten et al. 1980, Eil & Edelson 1984). Similarly, flutamide binds to androgen receptors, and it has been used to treat hirsutism alone or combined with OCs (Moghetti et al. 2000). Finasteride has also been used for the treatment of hirsutism. It inhibits the conversion of T to DHT and thereby decreases the effect of DHT on hair follicles (Rittmaster 1997). The antifungal agent, ketoconazole has also been shown to have some antiandrogenic effects in cases of hirsutism (Gokmen et al. 1996).

Metformin, which is the best documented and most widely used insulin sensitizer, has several mechanisms of action that result in improvement of hyperinsulinaemia. It suppresses hepatic glucose output (Stumvoll et al. 1995), decreases abdominal fat as reflected in a decrease in the waist/hip ratio (WHR) (Morin-Papunen et al. 2003) and decreases serum FFA levels (Perriello et al. 1994, Wiesenthal et al. 1999) as well as FFA uptake in peripheral tissues, favouring insulin-mediated glucose utilization (Abbasi et al. 1997, Abbasi et al. 1998). Metformin has also been shown to reduce androgen levels in women with PCOS, probably through decreases in BMI and insulin levels (Morin-Papunen et al. 2000).

Weight loss as a result of a low calorie diet or medical treatment has also been shown to decrease serum androgen levels (Longcope et al. 2000). However, this change is probably mediated through decreased insulin levels (Volek et al. 2002, Kaukua et al. 2003).

2.3 Aging and endocrine functions

2.3.1 Ovaries

2.3.1.1 Ovarian follicle reserve

At birth there are 1–2 million follicles in the human ovary, but the time of the onset of puberty their number has decreased dramatically leaving only 300,000 to 500,000 follicles (Faddy et al. 1992). During the menstrual cycle a few hundred follicles are selected from the follicle stock to grow to early follicular stage, but only one (dominant follicle) is selected to continue to the ovulatory stage (Fig. 5). The development of primary follicle to the ovulatory stage lasts approximately 200 days (Gougeon 1996). During the whole of woman’s reproductive life around 400 follicles will eventually ovulate, while the remainder die through programmed cell death (apoptosis) (Hsueh et al. 1994). The decline of both quantity and quality of the oocyte/follicle pool determines the age-dependent loss of female fertility. The number of small antral follicles in both ovaries, as measured by ultrasonography, is clearly related to reproductive age and is thought to reflect the size of the remaining primordial follicle pool (Scheffer et al. 1999, Scheffer et al. 2003).
Fig. 5. The various follicle stages in relation to follicle diameter and the phase and time of growth in women with normal menstrual cycles. Modified from Gougeon et al. (Gougeon 1996)

The follicle pool is decreased dramatically by age of 37–38 years and by the time of menopause (in Western populations approximately at the age of 51 years), the whole follicle stock has run out (Fig. 6).

Fig. 6. The decreasing follicle pool and corresponding reproductive events. Modified from te Velde et al. (te Velde et al. 1998b)
2.3.1.2 Ovarian steroid production

According to the prevailing concept, the period of optimal fertility, reflected in pregnancy and infertility rates, lasts until the age of about 30 and decreases gradually thereafter (van Noord-Zaadstra et al. 1991). Considering that the transition from regular to irregular menstrual cycles takes 6–7 years, regardless of age at menopause (den Tonkelaar et al. 1998) and that subfertility occurs even earlier, it is obvious that the decrease in ovarian hormonal environment starts at a fairly young age (Fig. 7). There are few studies concerning the timing of the decline in androgen levels with age in women and the current evidence suggests that the fall in androgens is not as clear as the sharp fall in circulating E2 concentrations at menopause (Labrie et al. 1997). For example, the results of some studies suggest that the fall in T levels begins as early as during the third decade with a gradual decline thereafter (Zumoff et al. 1995). On the other hand, some investigators have reported a change in androgen levels just before menopause (Longcope 1998) or a lack of change in T levels across the menopausal transition (Burger et al. 2000).

Data as regards ovarian E2 secretion with age are conflicting. However, it is well recognized that oestrogen levels decrease dramatically during menopause (Rannevik et al. 1995, Burger 1996).

There are only a few studies concerning the age-related androgen levels in women with PCOS. According to the results of one study total and non-SHBG-bound T levels decrease with age, implying that hyperandrogenism may improve before menopause in these women (Winters et al. 2000).

Fig. 7. 1) Cumulative age at menopause, 2) age at transition from regular to irregular cycles, 3) the end of fertility, 4) the beginning of subfertility. Modified from te Velde et al. (te Velde et al. 1998a)
2.3.1.3 Menopause

At menopause, at around the age of 50 years, the number of follicles is reduced to almost zero and the menstrual cycles stop. There is wide hereditary and individual variation in the time of menopause ranging from approximately 40 to 60 years (de Bruin et al. 2001), Fig. 7). Lifestyle factors also affect the time of menopause. Cigarette smoking, malnutrition and low economic status have been reported to lead to earlier menopause (Torgerson et al. 1994, van Noord et al. 1997). There has also been a debate whether or not OCs affect the timing of menopause (van Noord et al. 1997, de Vries et al. 2001). Occasionally the follicle stock diminishes faster than normal, causing premature menopause. If menopause occurs at the age of 40–45 it is considered "early" and occurs in about 5% of women. If amenorrhoea occurs prior to the age of 40 years it is considered as premature ovarian failure (POF). Only about 1% of women experience POF, and there are several factors that result in the loss of ovarian follicles. Depending upon the age at diagnosis, the most probable causes of POF are genetic, autoimmune, or idiopathic disturbances.

The link between follicle pool and the time of menopause has been seen in clinical practice as the women with low numbers of retrieved oocytes in the first IVF treatment are more likely to become postmenopausal at an early age than women with a higher number of retrieved oocytes (de Boer et al. 2002).

Even though there is a gradual age-related decrease in ovarian androgen production and a dramatic decrease in E2 levels during menopause, ovarian A and T production continues after menopause (Longcope 1986). Oophorectomy in postmenopausal women decreases T levels by 50%, which confirms the ovarian contribution to total androgen production in older age. Postmenopausal A and T production are at least partly under pituitary control, since GnRH-a or antagonist treatment has been shown to decrease A and T levels by 30% (Dowsett et al. 1988, Andreyko et al. 1992, Rabinovici et al. 1992).

2.3.1.4 Ovarian reserve tests

Ovarian reserve tests are thought to reflect indirectly the remaining follicle pool, and they are used in clinical practice to identify women who have a decreased probability of pregnancy (Burkman 1995, Broekmans et al. 1998).

In the GnRH-a test a high dose of GnRH-a causes extensive release of pituitary LH and FSH and consequently results in increased synthesis of androgens and E2 within 24 hours. The clomiphene citrate (CC) challenge test has also been used to investigate the ovarian reserve (Gulekli et al. 1999). Clomiphene citrate causes rises in ovarian E2 and inhibit B levels leading to a decrease in FSH levels through a negative feed back system. Decreased FSH suppression indicates a decreased ovarian reserve. Human chorionic gonadotrophin (hCG) is physiologically equivalent to pituitary LH, although its half-time in the blood circulation is longer. It can be used to evaluate the ovarian hormonal capacity, especially theca cell function. In addition counting of ovarian follicles by ultrasonography appears to correlate well with the ovarian follicle reserve (Loverro et al. 2003, Toner 2003).
2.3.2 **Markers of ovarian aging**

2.3.2.1 **Gonadotropins**

There are several hormonal markers that have been used to assess the decrease in ovarian follicle pool and endocrine function. One of the first signs of ovarian aging is the rise in serum FSH levels probably as a result of decreased feed-back inhibition by the ovaries. According to the results of one study the increase in FSH levels is observed before the age of 30 years (Ahmed Ebbiary *et al.* 1994), whereas other investigators have found elevated FSH levels between 30–40 years of age or in even in older women (Lee *et al.* 1988, Klein *et al.* 1996b). Serum LH levels also increase with age, but later than FSH levels (Lenton *et al.* 1988).

2.3.2.2 **Antimüllerian hormone**

Antimüllerian hormone (AMH), also known as Müllerian inhibiting substance/factor (MIS/MIF), is a member of the transforming growth factor-β (TGF-β) family. In males AMH is produced by Sertoli cells and it causes regression of the Müllerian ducts, which is a requirement for normal sex differentiation (Allard *et al.* 2000). In females, AMH is mainly secreted by the granulosa cells of ovarian small follicles (Fig. 8). Until puberty the serum levels of AMH are negligible but they increase thereafter to levels comparable to those in men, probably as a result of follicular growth, and they remain detectable until the end of ovarian activity (Vigier *et al.* 1984, Hudson *et al.* 1990, Josso *et al.* 1993, Young *et al.* 1999). The expression of AMH has been demonstrated in granulosa cells, and its receptors have been found both in granulosa and in theca cells by *in situ* hybridization (Baarends *et al.* 1995).

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**Fig. 8.** Action of AMH in the ovary according to *in vivo* and *in vitro* studies. AMH, which is produced by small ovarian follicles, has two major roles of action in the postnatal ovary. 1) It inhibits the initial follicle recruitment of follicles from primordial follicle pool and 2) decreases the sensitivity of ovarian follicles to FSH. Modified from Durlinger *et al.* (Durlinger *et al.* 2002).
The results of earlier studies have suggested that AMH may play an important role in follicle recruitment possibly by decreasing the sensitivity of ovarian follicles to FSH and by inhibiting the initiation of FSH-induced follicle growth, selection of the dominant follicle and thereby follicle pool depletion (di Clemente et al. 1994, Durlinger et al. 1999, Durlinger et al. 2002, Seifer et al. 2002)(Fig. 8). This may indicate that lack or excess of AMH secretion could be associated with abnormal follicular development and in thereby disturbances in reproductive functions.

Serum levels of AMH have been shown to be correlated with the number of small antral follicles (de Vet et al. 2002, van Rooij et al. 2002, Fanchin et al. 2003, Laven et al. 2004). Moreover, since there is a negative correlation between serum AMH concentration and age (Laven et al. 2004), it has been suggested that the AMH level could reflect the size of remaining follicle pool in women (van Rooij et al. 2002).

Women with PCOS have been shown to have 2- to 3-fold higher serum AMH levels than healthy women (Cook et al. 2002, Pigny et al. 2003, Laven et al. 2004), which may be a result of a larger pool of small follicles in PCOS subjects (Pigny et al. 2003). The decrease of serum AMH levels with age has also been observed in women with PCOS (Laven et al. 2004).

2.3.2.3 Inhibin B

Inhibin B, a dimeric glycoprotein of the TGF-β family, is produced by the granulosa cells of small antral follicles. According to negative correlation between follicular fluid/early follicular phase serum FSH and inhibin B levels (Groome et al. 1996), inhibin B has been suggested to inhibit FSH secretion. Since follicular phase inhibin B levels correlate inversely with age (Danforth et al. 1998) and since some investigators have reported a decrease in inhibin B levels as early as after the age 30 (Klein et al. 1996c, Welt et al. 1999), inhibin B has been used as a marker of ovarian aging. The age-related decrease in inhibin B levels can be explained by the crucially diminished ovarian follicle pool and granulosa cell number, and the decrease may also contribute to the age-related increase in FSH levels (Danforth et al. 1998, Welt et al. 1999).

Despite the fact that several investigators have reported large number of small antral and preantral follicles in women with PCOS, only a few have demonstrated increased inhibin B levels in these women (Anderson et al. 1998, Lockwood et al. 1998, Cortet-Rudelli et al. 2002). However, since several investigators have observed a negative correlation between the level of inhibin B and BMI (Pigny et al. 2000, Cortet-Rudelli et al. 2002), it has been suggested that BMI-related factors such as insulin or LH could suppress inhibin B levels in women with PCOS (Welt et al. 1999, Cortet-Rudelli et al. 2002).

2.3.3 Adrenals

Dehydroepiandrosterone sulphate, which is the only androgen originating almost entirely from the adrenals, has been used to assess adrenal androgen production. In females levels
of DHEAS increase markedly between the ages 6 and 14 years, peak at the age of 20 years and decrease gradually thereafter, being 70% lower by the age of 70 years when compared with those in women aged 30 years (Orentreich et al. 1984, Rozenberg et al. 1988). Even though women with PCOS have elevated serum adrenal androgen levels, an age-related decline in DHEAS levels has also been shown to occur in hyperandrogenic women (Moran et al. 1999)(Fig. 9). Similarly to ovarian A and T production (Judd et al. 1974, Longcope 1986), adrenal androgen production remains detectable after menopause in the end of female’s life (Laughlin & Barrett-Connor 2000). In contrast to adrenal androgen levels, cortisol levels have been shown to increase to some extent after menopause (Laughlin & Barrett-Connor 2000).

Fig. 9. Serum DHEAS levels vs. age in women with hyperandrogenism (Moran et al. 1999).
3 Purpose of the present study

In females age-related changes in basal serum gonadotropin, T and E2 levels have been studied extensively. However, data regarding the ovarian hormonal reserve and steroid secretion capacity during the reproductive life of healthy women as well as in women with PCOS are scarce or lacking. To study the age-related hormone profile in more detail, hCG tests were used to mimic the action of pituitary LH.

Since women with PCOS are known to have elevated levels of serum LH and also markedly increased adrenal androgen levels, it was of interest to study whether LH/hCG, in addition to ACTH, regulates androgen synthesis in the adrenals and whether these typical features of PCOS could be related to each other.

Several hormonal markers have been used to assess age-related changes in ovarian function, but owing to variable results none of them has been shown to be sensitive enough and optimal for clinical use. Therefore one of the aims of the present study was to test new markers which could better reflect the ovarian follicle pool and could be used to distinguish between normal and polycystic ovaries.

Specific aims of the study:

1. To study the age-related changes in ovarian androgen production in healthy women of fertile age by using hCG test.
2. To investigate whether the pronounced androgen secretion in women with PCOS persists throughout reproductive life and whether a similar decrease in androgen secretion capacity, as observed in healthy women, also occurs in women with PCOS.
3. To study whether adrenal androgen secretion is under the control of pituitary LH and whether this relationship could offer an explanation for the excessive serum adrenal androgen levels coinciding with high serum LH concentrations in women with PCOS.
4. Since studies I and II demonstrated that ovarian endocrine aging starts as early as before the age of 30 years, one of the purposes was to investigate the age-related profile of hormonal markers that could best reflect the decrease of ovarian follicle number and hormonal reserve. It was of particular interest to assess whether AMH, a new marker of the ovarian follicle pool, could be useful for evaluation of ovarian aging, diagnosis of PCO/PCOS and assessment of medical treatment responses.
4 Subjects and methods

4.1 Subjects

4.1.1 Regularly menstruating women (Studies I, III and IV)

Forty-four healthy women of fertile age (aged 20–44 years) participated in these studies. Body mass index, calculated as the ratio of weight (kg) to height (m²), ranged from 19.0 to 31.8 kg/m². All these volunteers exhibited regular cycles, defined as cycle with intermenstrual intervals of 21–35 days and inter-cycle lengths of ≤ 7 days. The follicular phase was confirmed by measuring serum progesterone (P) levels at the time of examinations. Furthermore, normal ovarian structure was confirmed by transvaginal ultrasonography. Six subjects had earlier been diagnosed as having mild or moderate endometriosis, which did not affect the ovaries, and they had not received any medical treatment for endometriosis. One subject had medication for depression and one used antihistamines for allergy (studies I and III). Otherwise the subjects did not have any medication and a break of at least two months was required in use of OC pills before participating in the studies. Despite the above-mentioned conditions, the group will be referred to as “healthy women” in the Results and Discussion section.

4.1.2 Postmenopausal women (Study III)

Six oophorectomized postmenopausal women (aged 47–59 years, BMI 25.5–34.4 kg/m²) volunteered for study III. They were all on oestrogen replacement therapy (ERT, oral or transdermal oestradiol). Two subjects had antihypertensive medication. One woman on simvastatin treatment for hyperlipidaemia forgot to inform the research team about her medication. Her androgen levels did not differ from those of the other subjects and therefore her results were included in the analyses.
4.1.3 Women with PCOS (Studies II and IV)

The PCOS group consisted of 65 women (aged 16–44 years, body mass index, 18–44 kg/m²). All these women had oligomenorrhea or amenorrhea. A cycle was considered oligomenorrhoic if the intermenstrual interval was ≥ 36 days. The subject was considered amenorrhoic if the intermenstrual interval was > 6 months. Other inclusion criteria were hyperandrogenism (hirsutism score > 7 according to Ferriman & Gallwey (Ferriman D 1961), acne or serum testosterone ≥ 2.7 nmol/L) and PCO observed in transvaginal ultrasonography (at least 8 follicles of 3–8 mm diameter in one plane in one ovary). One woman had medication for bronchial asthma and two used antihistamines for allergy. The subjects were otherwise healthy and used no regular medication (including OC pills) at the time of the examinations. Prior to serum sampling, progestin treatment (dydrogesterone, 10mg/day for 10 days) was used to induce menstrual bleeding in subjects with oligomenorrhea (if the menstrual interval was >2 months) or amenorrhea.

4.2 Study design

4.2.1 Serum sampling and laboratory methods

All women of fertile age were examined and serum samples collected at the follicular phase of the menstrual cycle (between cycle days 1 and 5, in metformin study between cycle days 1-7) after spontaneous or progestin-induced menstrual bleeding. The postmenopausal oophorectomized women were examined at any convenient time. All serum samples were fasting samples taken between 07.00 and 10.00 a.m. They were frozen at -20°C until analyzed. Details of the assays are given in Table 2. All assays were performed according to the instructions of the manufacturers.
Table 2. Characteristics of the assays used.

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<tr>
<th>Analyte</th>
<th>Method</th>
<th>Sensitivity</th>
<th>Coefficient of intra-assay variation (%)</th>
<th>Coefficient of inter-assay variation (%)</th>
<th>Reference range in follicular phase</th>
</tr>
</thead>
<tbody>
<tr>
<td>S-T</td>
<td>LIA</td>
<td>0.35 nmol/L</td>
<td>4.0</td>
<td>5.6</td>
<td>0.4-2.7 nmol/L</td>
</tr>
<tr>
<td>S-P</td>
<td>LIA</td>
<td>0.48 nmol/L</td>
<td>3.7</td>
<td>5.4</td>
<td>0.48-4.4 nmol/L</td>
</tr>
<tr>
<td>S-E2</td>
<td>RIA</td>
<td>20 pmol/L</td>
<td>5.7</td>
<td>6.4</td>
<td>40-260 pmol/L</td>
</tr>
<tr>
<td>S-Cortisol</td>
<td>RIA</td>
<td>5.0 nmol/L</td>
<td>4.0</td>
<td>4.3</td>
<td>150-650 nmol/L</td>
</tr>
<tr>
<td>S-DHEA</td>
<td>RIA</td>
<td>0.1 nmol/L</td>
<td>6.5</td>
<td>7.9</td>
<td>1.7-36 nmol/L</td>
</tr>
<tr>
<td>S-DHEAS</td>
<td>RIA</td>
<td>0.03 μmol/L</td>
<td>5.3</td>
<td>7.0</td>
<td>1.0-14 μmol/L</td>
</tr>
<tr>
<td>S-A</td>
<td>RIA</td>
<td>0.07 nmol/L</td>
<td>5.0</td>
<td>8.6</td>
<td>0.7-16 nmol/L</td>
</tr>
<tr>
<td>S-17-OHP</td>
<td>RIA</td>
<td>0.2 nmol/L</td>
<td>5.0</td>
<td>5.4</td>
<td>0.3-3.6 nmol/L</td>
</tr>
<tr>
<td>S-FSH</td>
<td>FIA</td>
<td>0.05 IU/L</td>
<td>3.8</td>
<td>4.3</td>
<td>2-10 IU/L</td>
</tr>
<tr>
<td>S-LH</td>
<td>FIA</td>
<td>0.05 IU/L</td>
<td>4.9</td>
<td>6.5</td>
<td>2-10 IU/L</td>
</tr>
<tr>
<td>S-AMH</td>
<td>EIA</td>
<td>0.7 pmol/L</td>
<td>5.3</td>
<td>8.7</td>
<td>12 ± 19 pmol/L*</td>
</tr>
<tr>
<td>S-Inhibin B</td>
<td>ELISA</td>
<td>&lt;15 pg/mL</td>
<td>5.0</td>
<td>5.4</td>
<td>5-200 pg/mL</td>
</tr>
</tbody>
</table>

*(mean ± SD)

4.2.2 Human chorionic gonadotropin test (Studies I, II and III)

The hCG test was used to assess the ovarian capacity to produce androgens and was performed 1–5 days after spontaneous or progestin-induced menstrual bleeding. Fasting blood samples were collected before a single intramuscular (i.m.) injection of 5000 IU hCG (Pregnyl® 5000 IU, N.V. Organon, Oss, Holland) between 07.00 and 10.00 a.m. and thereafter at 24, 48, 72 and 96 hours. The study protocol was simplified in study III, in that the 72 h time point was not collected/analyzed. To measure the hCG response, the area under the curve (AUC) 0–96 h (Fig. 10) was calculated by trapezoidal method (Altman 1991).
Fig. 10. Example of area under the curve (AUC).

To investigate the effect of LH/hCG on adrenal androgen production, serum samples for assay of DHEA, DHEAS and cortisol were taken during the hCG test. Furthermore, hCG tests were performed in six women (aged 21–39 years) with peritoneal endometriosis before and after 3 months of GnRH-a treatment (Enanton®, Depot 3,75mg, Laboratoires Cassenne Osnay, Cerny-Pontoise Cedex, France), as well as in six oophorectomized women (aged 47–59 years) before and after cessation of ERT.

4.2.3 Metformin treatment (Study IV)

Serum samples for assay of AMH were collected before and at 3 and 6 months of metformin treatment (metformin hydrochloride, Diformin, Leiras, Finland: 500 mg ×2 for 3 months, then 1000 mg ×2 for 3 months).

4.2.4 Vaginal ultrasonography

The vaginal ultrasonography was performed in all study subjects to confirm either normal ovarian structure or PCO. A diagnosis of PCO was made when 8 or more follicles (2–8 mm in diameter) were observed in one plane in one ovary. The follicle count was calculated as the mean value of the follicle number measured in each ovary. Ovarian volume was measured as the volume of an ellipsoid: 0.523 × length × width × thickness (Robert et al. 1995). Ultrasonography was carried out with Toshiba equipment (Toshiba SSSA-270A, Toshiba Co., Tokyo) with a 6 MHz transvaginal probe.
All data were analyzed by using the Statistical Program for Social Science (SPSS Inc., Chicago, IL, USA). Huynh-Feldt's correction was used in study I to measure significance within a study group and also to determine whether hCG stimulation differed between the groups. To compare serum hormone levels and ovarian responses to hCG (AUCs) between different age groups at each time point in studies I, II and III, the independent samples t-test was used as a post hoc test for normally distributed variables and the Mann-Whitney test for variables with skewed distribution. In studies I, II and IV, Pearson’s (r, normal distributions) and Spearman’s (skewed distributions) correlation coefficients were used to investigate the correlation between two factors. In study IV the analysis of variance (ANOVA) for repeated measures was used to analyze the impact of 6 months of metformin treatment on serum AMH levels, follicle number and volume. The Wilcoxon unpaired test was used for variables with persisting skewed distribution after log transformation. In studies II and IV multiple linear regression analysis and ANOVA were used to adjust the impact of BMI on the hormonal changes/differences. The limit of statistical significance was set at $p \leq 0.05$ in all studies.
5 Results and Discussion

5.1 Androgen secretion in women

5.1.1 Ovarian androgen secretion in healthy women

Pituitary LH is thought to regulate ovarian theca cell androgen production, whereas FSH promotes the aromatization of androgens to estrogens in granulosa cells (Fig. 11). The results of the present studies support the concept of the two-cell-two-gonadotrophin model of ovarian oestrogen biosynthesis, since administration of hCG, an analogue of pituitary LH, resulted in release of ovarian androgens. A significant increase in E2 levels after hCG injection was also observed. The increase in serum E2 concentration in response to hCG may simply be a result of increased secretion of E2 precursors. On the other hand, an alternative explanation for this phenomenon could be LH/hCG-induced aromatase activity as shown in earlier in vitro studies (Tapanainen et al. 1991). The responses of A, T and E2 to hCG appeared to be biphasic. The first burst may represent the release of storage hormones to the circulation and the second burst may be the result of de novo synthesis of these steroids (Fig 12).
Fig. 11. A possible mechanism of LH action/hCG stimulation in ovary.

According to the results of study III, the contribution of the ovaries to total 17-OHP, A and T secretion is around 30%, since GnRH-a treatment decreased the basal 17-OHP concentration by 27%, that of A by 18% and that of T by 38%. Furthermore, 17-OHP, A and T responses to hCG decreased by around 30% after GnRH-a treatment. These findings support the results of previous studies showing that 25–50% of T and around 50% of A are of ovarian origin (Longcope 1986, Morley & Perry 2003). However, since the concentrations of A were decreased by only 18% with GnRH-a treatment, it could be that ovarian contribution to A secretion is less significant than previous studies have suggested. This may also be a result of the fact that the treatment with GnRH-a did not result in complete suppression of ovarian hormone secretion. Androgen levels in oophorectomized women were significantly decreased when compared with those in young women (17-OHP -57%, A -46% and T -25%), although some of the decrease may have been a result of adrenal aging, since the oophorectomized women were postmenopausal. Serum E2 levels decreased markedly (by around 70%) after both GnRH-a treatment and oophorectomy, showing that E2 is mainly produced by the ovaries.
Fig. 12. Serum concentrations of 17-OHP, A, T and E2 during 5 days of hCG stimulation in young healthy women (20–44 years, \( n = 44 \)), \( p < 0.05 \) at all time points when compared with 0h.

### 5.1.2 Ovarian androgen secretion in women with PCOS

Hyperandrogenism is a typical feature of PCOS, but the mechanisms behind this condition are not clear. The results of study II demonstrated that the serum basal levels of A and T were significantly higher in women with PCOS than in healthy controls (Fig. 13). Similarly, the hCG-stimulated androgen levels were elevated (shown as AUC, Fig. 14), implying that the number or the activity/responsiveness of theca cells is increased in women with PCOS. This hypothesis is supported by the results of other studies indicating increased theca cell activity in women with PCOS (Gilling-Smith et al. 1994, Gilling-Smith et al. 1997, Nelson et al. 1999, Nelson et al. 2001).
Basal serum E2 levels were slightly but significantly increased in women with PCOS when the subjects were analysed as one group, although after setting the age division at 30 years this difference was not observed (study II). Other studies have also reported elevated basal E2 levels in women with PCOS (Barnes 1998, Rosenfield 1999), and various mechanisms have been suggested to explain this. One cause could be an increased ovarian response to FSH possibly as a result of a larger number of FSH-sensitive small antral follicles in women with PCOS (Van Der Meer et al. 1998), or increased granulosa cell sensitivity to FSH (Coffler et al. 2003). Moreover, increased
aromatase activity could also be one of the explanations (la Marca et al. 2002). The mild hyperestrogenism might also be a result of increased peripheral conversion of androgens to E2 in adipose tissue, since women with PCOS are often more obese than the control women (Franks 1995, Conway 1996). However, all the hormonal differences between women with PCOS and the control subjects remained after adjustment for BMI, suggesting that the obesity of PCOS subjects is not the only explanatory factor. The response of E2 to hCG was comparable to that in healthy women (Fig. 15).

Fig. 15. Basal serum E2 levels and the response to hCG stimulation shown as AUC 96h in healthy women and in women with PCOS.

5.1.3 Adrenal androgen secretion

The results of study III indicate that LH/hCG does not have a major role in adrenal steroid production in healthy women even though some women with PCOS have excessive adrenal androgen production with concomitantly elevated serum LH levels. Although the serum levels of DHEA, DHEAS and cortisol remained unchanged after a single injection of hCG (Table 3.), a possible stimulatory effect of chronically elevated LH on adrenal androgen secretion cannot be excluded. In fact, the studies carried out on transgenic female mice with constitutively high LH levels showed elevated corticosteroid production in these animals (Kero et al. 2000). The same study also revealed LHR mRNA expression in the adrenal glands as well as an increased adrenal response to ACTH in the presence of hCG. It is also noteworthy that short-term incubation of adrenal cells with hCG caused a significant increase in cortisol and A secretion (O'Connell et al. 1994), suggesting that LH/hCG could affect adrenal steroid production.

Despite the fact that short-term hCG treatment did not affect adrenal androgen synthesis, there are data implying that regulatory factors other than ACTH may also
control adrenal androgen secretion. For example, during adrenarche adrenal androgen production rises and reaches adult levels without a concomitant increase in ACTH levels (Apter et al. 1979).

Table 3. Basal serum DHEA, DHEAS and cortisol levels in women aged 21-39 years and in postmenopausal oophorectomized women.

<table>
<thead>
<tr>
<th>Hormone</th>
<th>regularly menstruating women</th>
<th>postmenopausal oophorectomized women</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(n = 6)</td>
<td>(n = 6)</td>
</tr>
<tr>
<td>DHEA (nmol/L)</td>
<td>35.83 ± 4.24</td>
<td>16.30 ± 3.49</td>
</tr>
<tr>
<td>DHEAS (µmol/L)</td>
<td>4.91 ± 0.77</td>
<td>2.63 ± 0.53</td>
</tr>
<tr>
<td>cortisol (µmol/L)</td>
<td>0.56 ± 0.10</td>
<td>0.41 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>basal</td>
<td>max during hCG</td>
</tr>
<tr>
<td></td>
<td>33.00 ± 12.11</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>4.81 ± 1.57</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>0.48 ± 0.19</td>
<td>NS</td>
</tr>
</tbody>
</table>

Mean ± SE; NS non-significant

The present results indicate that DHEA and DHEAS are mainly of adrenal origin since their concentrations remained unchanged in hCG tests, whereas 17-OHP, A, T and E2 levels increased significantly (Fig 14). Similarly treatment with GnRH-a had no effect on serum DHEA, DHEAS or cortisol levels, whereas ovarian steroid concentrations decreased markedly. Levels of both DHEA and DHEAS were 50% lower (DHEA: \( p = 0.005 \), DHEAS: \( p = 0.035 \)) and cortisol levels were 20-30% lower (NS) lower in postmenopausal oophorectomized women when compared with those in young healthy women (Table 3). The decreased androgen levels were probably a result of the postmenopausal status of the oophorectomized women, since adrenal androgen production has been shown to decrease with age (Orentreich et al. 1984, Parker et al. 2000). The decrease in cortisol levels observed in study III was not statistically significant, this being in line with the results of a previous study indicating unchanged cortisol levels in pre- vs. postmenopausal women (Parker et al. 2000).

5.2 Gonadotropin secretion in healthy women and in women with PCOS

Circulating gonadotropin levels were decreased dramatically during 3 months of GnRH-a treatment (LH -87%, FSH -47%) in healthy women. In oophorectomized women, LH and FSH levels were significantly higher than in young women (LH +726%, FSH +1218%), obviously as a result of a lack of ovarian negative feedback. Mean LH and FSH values in these women were 40.1 ± 7.5 IU/L and 66.8 ± 8.3 IU/L four weeks after cessation of ERT. The levels were comparable to those observed in other study carried out among naturally postmenopausal women (Heikinheimo et al. 2000), although some investigators have reported even higher LH and FSH concentrations (Hall et al. 2000, Gill et al. 2002).

Inappropriate gonadotrophin secretion in women with PCOS was first reported by Yen et al. (Yen et al. 1970). Women with PCOS have been shown to have lower FSH levels and higher LH levels than healthy women (Holte et al. 1994, Taylor et al. 1997) and thereby also an increased LH/FSH ratio. The results of study II support this since serum
FSH levels were decreased and the LH/FSH ratio was increased in women with PCOS when compared with healthy women (Table 4). Levels of LH tended to be higher in women with PCOS, but the difference did not reach statistical significance (Table 4).

Table 4. LH, FSH and the LH/FSH ratio in healthy women (study I, n = 44) and in women with PCOS (study II, n = 42).

<table>
<thead>
<tr>
<th>Hormone</th>
<th>Control</th>
<th>PCOS</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>LH (U/L)</td>
<td>5.37 ± 0.5</td>
<td>5.94 ± 0.4</td>
<td>NS</td>
</tr>
<tr>
<td>FSH (U/L)</td>
<td>6.93 ± 0.7</td>
<td>5.18 ± 0.2</td>
<td>0.014</td>
</tr>
<tr>
<td>LH/FSH</td>
<td>0.82 ± 0.0</td>
<td>1.18 ± 0.1</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Mean ± SE, NS non significant.

There are several studies and speculations concerning the role of elevated LH levels on hyperandrogenism in women with PCOS. The abnormal LH secretion may result from inappropriate GnRH secretion/response in women with PCOS causing increased in LH pulse amplitude and frequency (Venturoli et al. 1988, Taylor et al. 1997). These women have also been shown to have an enhanced response to GnRH-a treatment (Barnes 1989). The results of some studies have implied that excessive LH levels could cause increased androgen synthesis in theca cells, although others have suggested that androgen synthesis is increased in women with PCOS independently of gonadotropin secretion (Shoham et al. 1992, Gilling-Smith et al. 1994, Gilling-Smith et al. 1997). Moreover down-regulation of LHRs may be abnormal in women with PCOS causing increased levels of active receptors and consequently increased stimulation of theca cells (Ehrmann et al. 1995, Rosenfield 1999).

In contrast to serum LH levels, FSH concentrations have been shown to be comparable (Gonzalez et al. 1991, Escobar-Morreale et al. 2001, Amer et al. 2002) or decreased (Barnes et al. 1989) in women with PCOS when compared to control women. The results of study II suggests decreased FSH concentration in women with PCOS (Table 4). The excessive production of E2 precursors (androgens) and slightly increased serum E2 levels, as well as increased follicle number, may explain the decreased serum FSH levels and increased LH/FSH ratio in women with PCOS (Table 4.). Altogether, the present results did not allow us to determine whether the inappropriate gonadotrophin secretion in women with PCOS is primary or secondary to inappropriate androgen secretion.

5.3 The ovary and aging

5.3.1 Healthy women

The ovarian hormonal environment changes with age as the ovarian follicular reserve diminishes through the mechanisms of apoptosis (Tilly 1996, Vaskivuo & Tapanainen 2003). According to Faddy et al. (Faddy et al. 1992) the decrease in follicle number
accelerates when the total count has fallen to 25,000 at the age of about 37.5 years. However, since female fertility has been shown to be already decrease before the age of 37.5 years (van Noord-Zaadstra et al. 1991), ovarian hormonal capacity would be expected to decrease even earlier.

The results of study I indicated that the ovarian capacity to synthesize androgens starts to decrease early, since basal serum 17-OHP and A levels were decreased in women over 25 years when compared with those in women of 25 years or younger (Table 5a). There was also a similar trend in serum T levels, although the decrease was not statistically significant. The ovarian capacity to secrete androgens (17-OHP and T) in response to hCG, was also decreased before the age of 30 years. Furthermore, the AUCs of 17-OHP and T at 96 h correlated negatively with age (17-OHP: $r = -0.427, p = 0.004$; T: $r = -0.354, p = 0.018$). The early decrease in androgen levels is supported by the results of a previous study, where decreased serum T levels were shown to occur as early as at the beginning of the third decade (Zumoff et al. 1995). The decrease in basal and hCG-stimulated serum androgen levels is most likely a result of a decreased number of follicles, although age-related changes in the whole hypothalamic-pituitary-ovarian axis may also contribute to changes in ovarian androgen production. Ovarian sensitivity to gonadotrophin stimulation may change with age, although our results do not allow us to confirm these hypotheses.

Differently from androgens, basal serum E2 levels remained unchanged even when the age division was set at 35 years (Table 5c). However, AUC E2 was significantly increased in women of >30 years compared with those of ≤30 years (Table 5b). There are several studies concerning age-related changes in serum E2 levels, but the results are conflicting. They have demonstrated decreased (Labrie et al. 1997, Broekmans et al. 1998), increased (Musey et al. 1987, Klein et al. 1996b, Kim et al. 1997) or unchanged (Furushashi et al. 1977, MacNaughton et al. 1992) E2 levels during aging. In our studies maximal E2 levels after hCG injection were reached later in women of >30 years compared to younger women (Fig. 19), which could reflect decreased numbers of granulosa cells in older women and/or smaller releasable stores of E2 precursors.

### Table 5. a. Serum basal and hCG stimulated (AUC) 17-OHP, A, T and E2 levels in relation to age division at 25 years (≤25 n=13, >25 n=31) in healthy women.

<table>
<thead>
<tr>
<th>Hormone (nmol/L)</th>
<th>Basal</th>
<th>AUC</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>≤25</td>
<td>&gt;25</td>
<td>p</td>
</tr>
<tr>
<td>17-OHP (nmol/L)</td>
<td>5.5 ± 0.7</td>
<td>3.8 ± 0.4</td>
<td>0.03</td>
</tr>
<tr>
<td>A (nmol/L)</td>
<td>10.0 ± 0.5</td>
<td>8.7 ± 0.5</td>
<td>0.03</td>
</tr>
<tr>
<td>T (nmol/L)</td>
<td>1.7 ± 0.2</td>
<td>1.3 ± 0.1</td>
<td>NS</td>
</tr>
<tr>
<td>E2 (nmol/L)</td>
<td>0.11 ± 0.02</td>
<td>0.12 ± 0.01</td>
<td>NS</td>
</tr>
</tbody>
</table>

Mean ± SE; NS non-significant
Table 5. b. Serum basal and hCG stimulated (AUC) 17-OHP, A, T and E2 levels in relation to age division at 25 years (≤30 n=20, >30 n=24) in healthy women.

<table>
<thead>
<tr>
<th>Hormone</th>
<th>Basal</th>
<th>AUC</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>≤30</td>
<td>&gt;30</td>
<td>≤30</td>
</tr>
<tr>
<td>17-OHP</td>
<td>4.9 ± 0.5</td>
<td>3.8 ± 0.4</td>
<td>NS</td>
</tr>
<tr>
<td>A (nmol/L)</td>
<td>10.0 ± 0.5</td>
<td>8.3 ± 0.6</td>
<td>0.02</td>
</tr>
<tr>
<td>T (nmol/L)</td>
<td>1.5 ± 0.1</td>
<td>1.3 ± 0.1</td>
<td>NS</td>
</tr>
<tr>
<td>E2 (nmol/L)</td>
<td>0.12 ± 0.01</td>
<td>0.13 ± 0.02</td>
<td>NS</td>
</tr>
</tbody>
</table>

Table 5c. Serum basal and hCG stimulated (AUC) 17-OHP, A, T and E2 levels in relation to age division at 35 years (≤35 n=28, >35 n=16) in healthy women.

<table>
<thead>
<tr>
<th>Hormone</th>
<th>Basal</th>
<th>AUC</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>≤35 (n=28)</td>
<td>&gt;35 (n=16)</td>
<td>≤35</td>
</tr>
<tr>
<td>17-OHP</td>
<td>4.3 ± 0.4</td>
<td>4.4 ± 0.6</td>
<td>0.04</td>
</tr>
<tr>
<td>A (nmol/L)</td>
<td>9.6 ± 0.4</td>
<td>8.1 ± 0.7</td>
<td>NS</td>
</tr>
<tr>
<td>T (nmol/L)</td>
<td>1.5 ± 0.1</td>
<td>1.2 ± 0.2</td>
<td>NS</td>
</tr>
<tr>
<td>E2 (nmol/L)</td>
<td>0.13 ± 0.01</td>
<td>0.11 ± 0.02</td>
<td>NS</td>
</tr>
</tbody>
</table>

Mean ± SE; NS non-significant

5.3.2 Women with PCOS

To study the age-related changes in ovarian steroid production in women with PCOS and to answer the question of whether or not the hyperandrogenism persists until late reproductive life in these women, basal and hCG-stimulated steroid levels were measured. According to the results of study II, androgen levels remained about 50% higher in women with PCOS until late reproductive age when compared with those in healthy women (Fig. 16). When the age division was set at 30 years, there were no significant differences in A and T levels within the PCOS group, but the levels were significantly higher than in control women (Fig. 16). This finding is parallel with that in an earlier study showing persistently high T levels in women with PCOS at late fertile age (Winters et al. 2000).
Fig. 16. Basal serum A and T levels in women with PCOS and in healthy women of ≤ 30 years and > 30 years of age.

Moreover, along with aging, the ovarian capacity to secrete androgens in response to hCG stimulation remained high in women with PCOS as shown by increased AUC A and T (Fig. 17). This confirms the findings of earlier studies, with increased androgen responses to hCG/LH and/or GnRH-a stimulation in women with PCOS (Ehrmann et al. 1992, Ibanez et al. 1996, Gilling-Smith et al. 1997, Levrant et al. 1997). The persistently high basal and hCG-stimulated androgen levels in women with PCOS suggest that the activity of theca cells and/or of ovarian stroma remains enhanced until late reproductive years.
Fig. 17. AUC 96 h of A and T in women with PCOS and in healthy women of ≤ 30 years and > 30 years of age.

Despite pronounced increase in basal and hCG-stimulated androgen levels in women with PCOS, a similar age-related decrease in the ovarian response to hCG as in healthy women was also observed in the PCOS group. AUC A was significantly lower in women of > 30 years than in women of < 30 years (AUC A: 1343.8 ± 96.0 vs. 1058.1 ± 66.0, p = 0.016) and a similar trend was also seen in AUC T (Fig. 17). Another study has also revealed an age-related decrease in both A and T levels in women with PCOS. Moreover, there was a significant negative correlation between age and AUC A and a similar trend in correlation of age and AUC T (Fig. 18). The non-significant decrease of AUC T may be explained by the large individual variation, and it is possible that it would have become significant with a higher number of subjects. On the basis of power analysis 172 subjects would have been needed to demonstrate this.
**Fig. 18.** Pearson’s correlation between age and AUC at 96 of A and T in women with PCOS (●, n = 42) and in healthy women (○, n = 44). The AUC values shown are mean values over 96 h.

**Fig. 19.** Stimulation of E2 by means of hCG in healthy women and in women with PCOS. When the age division was set at 30 years, serum basal and hCG-stimulated E2 levels were comparable in both age groups in women with PCOS, and the levels were also comparable with those observed in healthy women (Fig. 19 and Table 5).

The age-related decrease in ovarian endocrine function is most likely a result of follicle loss both in healthy ovaries and in PCOs. In fact, the follicle count observed in women with PCOS in study IV correlated negatively with age after adjustment for BMI (from \( p = 0.723 \) to \( p = 0.039 \)), which supports similar findings in studies on healthy ovaries (Faddy *et al.* 1992). Since the follicle count in women with PCOS seems to decrease with age, it would mean that the hormonal environment in these women might
also normalize before the end of fertility. This is supported by observations that in some women with PCOS menstrual cycles and ovarian morphology normalize with age (Koivunen et al. 1999, Bili et al. 2001). Even though the number of small antral follicles has been thought to reflect the size of the follicle pool, the possible postponement of menopause owing to an increased number/bigger reserve of follicles in women with PCOS cannot be predicted.

5.4 Markers of ovarian aging

5.4.1 Gonadotrophins

The age-related decrease of ovarian function is reflected by a significant change in the hypothalamic-pituitary-ovarian axis. Since E2, a product of granulosa cells, is one of the most important regulators of pituitary gonadotropin secretion, the decrease in serum E2 levels and the negative feedback system results in an increase in gonadotrophin levels. One of the first signs of ovarian hormonal aging is the rise in FSH levels, which was observed to increase as early as after the age of 25 years (Fig. 20). This finding was supported by the results of another study in which FSH levels were reported to increase as early as at the age of 29–30 years (Ahmed Ebbiary et al. 1994). Other studies have also reported an age-related increase in follicular phase FSH levels, although the time point when FSH starts to rise varies (Klein et al. 1996a, Kim et al. 1997, Broekmans et al. 1998).

Since serum E2 levels seem to remain at physiological levels until late reproductive years, it is tempting to speculate that the early rise in FSH concentration might compensate for decreasing ovarian function and sustain the unchanged E2 levels. Whether or not the elevated FSH levels occur simply because of the decrease of negative feedback caused by diminished E2 is not clear. Decreased secretion of inhibin B from granulosa cells would lead to increased FSH concentrations as a result of a decreased negative feedback effect of inhibin B on the pituitary. This finding is supported by results that have shown a negative correlation between concentrations of inhibin B and FSH (Klein et al. 1996a), although no such correlation was observed in the present studies. Interestingly, in women with PCOS serum FSH levels remained normal until the age of 44 years (Fig. 20). It is possible that the excessive androgen production or slightly elevated E2 levels in women with PCOS sustains normal FSH levels through a feedback mechanism. What happens to gonadotropin and steroid levels in menopausal transition in women with PCOS needs to be investigated in a prospective follow-up study.

Serum LH levels have also been shown to increase during aging, although later than FSH levels (Lenton et al. 1988, MacNaughton et al. 1992). The results of study I support this concept, since no significant change was observed in serum follicular phase LH levels in healthy women (Fig. 20).
5.4.2 Antimüllerian hormone

Serum AMH levels have been shown to correlate well with the remaining follicle number and age, and therefore the assay of AMH has been thought to be useful in assessment of the ovarian aging process (de Vet et al. 2002, van Rooij et al. 2002). In study IV a significant negative correlation was observed between serum AMH concentrations and age both in healthy women and in women with PCOS, which supports the observations concerning the relationship between age and AMH (Fig. 21). In addition, a positive correlation between serum AMH levels and follicle number in women with PCOS was observed (Fig. 22). After dividing the healthy women into two groups according to age by setting the age limit at 25 years, serum AMH levels were significantly lower in the older women. This suggests that the decline in AMH secretion starts considerably early. Serum AMH concentrations correlated positively with serum A and T levels in women with PCOS (A: \( r = 0.309, p = 0.014 \); T: \( r = 0.312, p = 0.012 \)), and a similar correlation was observed between AMH and A concentrations in healthy subjects (\( r = 0.480, p = 0.001 \)). These observations strengthen the results of previous studies (Pigny et al. 2003, Laven et al. 2004) and emphasize the importance of small ovarian follicles in the production of both AMH and androgens.
Fig. 21. Correlation between serum AMH concentration and age in healthy women (○, n = 44) and in women with PCOS (●, n = 65).

Fig. 22. Correlation between follicle number and serum AMH concentration in women with PCOS (●, n = 65)
A negative correlation between serum AMH and FSH levels (Fig. 23) was observed in healthy women, which has also been reported by other authors (Seifer et al. 2002, van Rooij et al. 2002, Fanchin et al. 2003, Pigny et al. 2003). However, when the two extreme values observed in control women were excluded from the statistical analyses the p-value decreased to p = 0.053. The fact that FSH has been shown to decrease the expression of AMH as well as that of its type II receptors in granulosa cells (Baarends 1995), implies that FSH contributes to serum AMH levels.

One group of investigators has reported a negative correlation between serum AMH and E2 concentrations in women with PCOS (Cook et al. 2002). This has been explained by an inhibitory effect of AMH on aromatase activity (Vigier et al. 1989) which could cause an increase in androgen levels and unchanged levels of E2 in women with PCOS (Laven et al. 2004). The present and previous data (Pigny et al. 2003) do not, however, support this concept since no correlation between AMH and E2 concentrations was observed in women with PCOS. In contrast to the results of an earlier study (Laven et al. 2004), no correlation was observed between serum AMH and LH concentrations, or between serum inhibin B and AMH levels in healthy women or in women with PCOS. The discrepancy between the present and previous studies might be explained by the fact that serum levels of LH and inhibin B change later than the levels of AMH.

The results of study IV support findings in previous studies showing that AMH levels are 2- to 3-fold higher in women with PCOS compared with healthy women (Cook et al. 2002, Pigny et al. 2003, Laven et al. 2004) (Fig. 21). Increased serum AMH concentrations in PCOS have been explained by the increased number of small ovarian follicles responsible for AMH secretion (Pigny et al. 2003). The positive correlation

Fig. 23. Pearson’s correlation between serum AMH and FSH concentration in healthy women (○, n = 44) and in women with PCOS (●, n = 65).
between AMH concentrations and follicle number in women with PCOS in the present study supports this concept. Whether AMH has a regulatory role in follicle development or whether it is a consequence of increased antral follicle number in women with PCOS is not clear.

Although AMH has been shown to inhibit initial follicle recruitment (Durlinger 1999) and FSH-stimulated follicle growth (Durlinger et al. 2002) in animal studies and cell culture conditions, the definitive role of AMH in the regulation of human follicle development remains to be investigated. As a new finding, the results of study IV demonstrated that the difference in AMH concentrations between healthy women and women with PCOS remains until late reproductive age. Despite the age-related decrease in AMH levels in both healthy subjects and those with PCOS, serum AMH levels were continuously high in women with PCOS. In fact, serum AMH levels became undetectable in the majority of control women by the age of 38 years, whereas in the majority of women with PCOS serum AMH levels were measurable or even high (Fig 21). Since AMH levels correlate well with age, androgen levels and follicle number, assay of AMH could be a useful tool to assess ovarian aging and to diagnose PCOS/PCOS. As a matter of fact, assays of AMH could even be more sensitive as a marker of ovarian small follicles than vaginal ultrasonography, since the smallest follicles are difficult to detect, especially in obese women.

Although the positive effect of metformin treatment on menstrual pattern, ovulatory function and hyperandrogenism in women with PCOS is widely documented (Velazquez et al. 1994, Nestler & Jakubowicz 1997, Morin-Papunen et al. 1998), studies concerning its effect on ovarian morphology/ultrasonographic appearance are few (Elter et al. 2002) and no data on serum AMH levels during metformin treatment have been reported before. The present results show, for the first time, that serum AMH levels, as well as the number of ovarian follicles and ovarian volume, decrease during metformin therapy (Table 6). Whether this is simply related to the decrease in follicle number and/or the improvement of hyperandrogenism or insulin action, remains to be studied. The possible role of AMH as one of the mediators of metformin action on follicle number/growth cannot be excluded.

**Table 6. The effect of metformin treatment on serum AMH levels, follicle number and ovarian volume in women with PCOS (n = 16).**

<table>
<thead>
<tr>
<th>Variables</th>
<th>0 months</th>
<th>3 months</th>
<th>p</th>
<th>6 months</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMH (pmol/L)</td>
<td>72.59 ± 11.5</td>
<td>66.14 ± 12.7</td>
<td>NS</td>
<td>66.81 ± 13.0</td>
<td>0.03</td>
</tr>
<tr>
<td>follicle number</td>
<td>11.13 ± 0.7</td>
<td>8.25 ± 0.4</td>
<td>&lt;0.001</td>
<td>7.88 ± 0.6</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>ovarian volume</td>
<td>7.46 ± 0.6</td>
<td>6.57 ± 0.5</td>
<td>NS</td>
<td>6.52 ± 0.6</td>
<td>0.01</td>
</tr>
</tbody>
</table>

Mean ± SE; NS non-significant

Altogether, even though serum AMH concentrations seem to correlate well with the number of ovarian small follicles and their androgen activity, owing to great individual variation in AMH concentrations, a prospective follow-up study with an adequate number of subjects would be needed to determine the reference ranges in healthy women and in women with PCOS. In addition, the impact of insulin, obesity and hormonal medication
on serum AMH concentrations should be investigated before AMH measurements can be considered for clinical use.

5.4.3 Inhibin B

Inhibin B levels have also been shown to decrease with age, although the decrease occurs later than that of AMH (MacNaughton et al. 1992, Klein et al. 1996c, Danforth et al. 1998, Welt et al. 1999). No correlation between serum inhibin B and age between the ages of 20 to 44 years was observed in study I. This was also the case in women with PCOS, although the levels were about 20% higher in women with PCOS when compared with control women (study II). Even though this was not statistically significant, other investigators have reported elevated inhibin B levels in women with PCOS (Anderson et al. 1998, Lockwood et al. 1998, Cortet-Rudelli et al. 2002), probably as a result of an increased number of small ovarian follicles in these women. Thus, the inhibin B level could be a useful marker of ovarian aging later, at the premenopausal stage.

5.5 Methodological considerations

The study protocols were designed carefully, but there are still factors that may have affected the results.

It is well known that the results of cross-sectional and non-prospective studies are difficult to interpret, and in the present study this especially concerns the impact of aging on hormonal parameters.

Furthermore, the menstrual phase/ovulatory status of women with oligo/amenorrhea was not determined by ultrasonography or hormone assays, and the follicular phase was determined on the basis of spontaneous or progestin induced menstrual bleeding. This protocol could have led to misinterpretation of the cycle phase and caused variation in the timing of studies. It is also possible that treatment with progestin could have affected the results since it has been shown to decrease follicular phase T, A and LH concentrations (Anttila et al. 1992). If this was the case the difference in hormone levels between control women and the women with PCOS would actually have been even more significant than observed.

The ovarian capacity to synthesize and secrete androgens in regularly menstruating women as well as in women with PCOS was estimated by using AUCs, which were determined on the basis of serum basal and hCG-stimulated hormone concentrations at 0, 24, 48, 72 and 96 hours in relation to hCG injection. The difference between hCG-stimulated and basal levels were not used, since the aim was to describe the total ovarian capacity to secrete androgens and not just the response to hCG.
6 Conclusions

1. A decline in ovarian endocrine function before the age of 30 is one of the first signs of ovarian aging. Despite a relatively large follicle pool in young women, the changes in ovarian hormonal environment occur much earlier than any clinical signs of ovarian aging such as menstrual irregularity or decreased fertility. Even though there is a decrease in androgen secretion capacity at fairly young age, serum E2 levels remain at physiological levels until the late reproductive years in healthy women, and compensatory mechanisms may be needed to maintain optimal oestrogen biosynthesis.

2. Basal serum levels of androgens and ovarian androgen secretion capacity are markedly increased and remain high throughout the reproductive years in women with PCOS. However, similarly to healthy women there was a decrease in ovarian capacity to synthesize and release androgens in response to hCG stimulation in women with PCOS. The elevated androgen levels were associated with a high LH/FSH ratio and increased serum AMH levels (2- to 3-fold higher than in healthy women). The persistently increased androgen production in PCOS was associated with normal or elevated serum E2 levels, and unchanged serum FSH concentrations.

3. Luteinizing hormone does not have a major role in the regulation of adrenal steroid synthesis in healthy women, although it is possible that long-term exposure to LH/hCG could affect adrenal function. The results of GnRH-a treatment and hCG stimulation tests in young healthy women indicate that the ovarian contribution to the synthesis of 17-OHP, A and T is 25–30%, and that the adrenals are the primary source of cortisol, DHEA and DHEAS.

4. Decreased serum AMH and increased FSH levels in the follicular phase are the first signs of ovarian aging, since these hormonal alterations were observed as early as before the age of 30 years in healthy women. Furthermore, AMH levels correlated negatively with age, showing that it could be a potential marker of the ovarian aging process. An age-related decrease in serum AMH levels was also observed in women with PCOS, although their AMH levels remained high until late reproductive years, whereas in most of the control subjects AMH levels became undetectable before the age of 38. The persistently high AMH levels in PCOS could serve as a tool to diagnose PCOs/PCOS. Since AMH is secreted by ovarian small follicles and therefore directly reflects the changes of ovarian follicle number, it may be a better marker of ovarian
aging than FSH, which only reflects the changes in ovarian hormonal environment. Since treatment with metformin decreased serum AMH levels as well as affecting ovarian morphology, assay of AMH may be used to assess medical treatment responses.
References


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