Polycystic ovary syndrome

Ovarian pathophysiology and consequences after the menopause

by

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Till min familj

Abstract

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Ovarian pathophysiology and consequences after the menopause

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The Polycystic Ovary Syndrome (PCOS) is an endocrine disorder affecting ~10% of women. It is characterized by oligo/anovulation, hyperandrogenism, and polycystic ovaries. PCOS is associated with acne, hirsutism, infertility, abdominal obesity, type 2 diabetes, hypertension and dyslipidemia, with the latter four being cardiovascular disease (CVD) risk factors.
The aims of the thesis were to study PCOS regarding ovarian pathophysiology and postmenopausal development concerning anthropometry, reproductive hormones, bone mineral density (BMD), fractures, CVD risk factors and events and mortality.
Ovarian (stroma and granulosa cells) expression in selected genes of PCOS/controls was analyzed by quantitative PCR, with special emphasis on inflammation. In the central stroma of PCOS ovaries, genes of five inflammation-related factors, one inflammation-related transcription factor and one growth factor were under-expressed. One growth factor and one coagulation factor were over-expressed. In the granulosa cells of the PCOS women, all of the differentially expressed genes were over-expressed (five inflammation-related, two coagulation-related, two growth factors, one permeability-related and one growth-arrest-related).
Thirty-five PCOS women (diagnosed 1956-65) and their 120 randomly allocated age-matched controls (from the WHO MONICA study, Gothenburg), were examined in 1987 regarding anthropometry, reproductive hormones, CVD risk factors, lifestyle factors, medication and medical history (via questionnaire) and for the present thesis re-examined in 2008 (mean age 70.3 years) with the same variables. BMD was assessed by single photon absorptiometry in 1992 and by dual energy x-ray absorptiometry at follow-up in 2008. The National Board of Health and Welfare Registry and the Hospital Registry provided information on morbidity and mortality.
The PCOS women still had higher free androgen index (FAI), but lower FSH than controls. Hirsutism, hypertension and hypertriglyceridemia were more common, but climacteric symptoms and hypothyroidism were less prevalent among the PCOS women. The higher waist/hip ratio among the PCOS women in 1987 could not be detected at follow-up, possibly due to an increase in hip circumference in the PCOS women and to an increase in weight among the controls. BMD, fractures, diabetes, CVD events, total mortality and cancer incidence were similar in the PCOS women and controls at follow-up.
In conclusion, the ovaries of the PCOS women showed differences in the expression of key proteins, with implications for PCOS-specific arrested folliculogenesis and OHSS risk. Late postmenopausal PCOS women were still hyperandrogenic and hirsute with persistent hypertension and hypertriglyceridemia. However, the incidence of fractures, diabetes, cancer, CVD morbidity and total mortality was similar to that of the general population. Differences in body composition had disappeared in the PCOS women compared with the controls during 21 years of follow-up.

Key words: body composition, bone mineral density, cardiovascular disease, fracture, gene-expression, menopause, mortality, ovary, polycystic ovary syndrome, reproductive hormones
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Svensk sammanfattning

Polycystiskt ovarie syndrom (PCOS) förekommer hos ungefär var tionde kvinna och karakteriseras av oregelbundna/uteblivna menstruationer, ökat antal omogna, tillväxtavstannade äggblåsor (folliklar) och förhöjda nivåer av manliga könshormoner (androgener). Detta kan ge acne eller ökad kroppsbehåring (hirsutism) och oönskad barnlöshet (infertilitet). PCOS är i åldrarna fram till klimakteriet förknippat med bukfetma, ökad risk för typ 2 diabetes, högt blodtryck samt förhöjda blodfetter. En ökad risk för hjärtkärlsjukdom kan därför föreligga men långtidsstudier saknas och dessutom är orsaken till PCOS fortfarande oklar.

Syftet med denna avhandling var att studera några av PCOS äggstockens möjliga patofysiologiska mekanismer och konsekvenserna av PCOS efter klimakteriet.

Vissa genuttryck studerades med hjälp av kvantitativ polymerase chain reaction i två olika typer av vävnader i prov från äggstockarna hos kvinnor i fertil ålder med PCOS jämfört med kontroll kvinnor. Det fanns skillnader i genuttrycket som var relaterade till inflammation, tillväxtfaktorer och blodlevringsförmåga samt för en permeabilitetsrelaterad och en tillväxthämmande gen mellan kvinnor med PCOS och friska kontroll kvinnor.

Vidare har i denna avhandling kvinnor med PCOS (n=35) följts under 21 år efter klimakteriet och jämförts med 120 jämnåriga, slumpvist utvalda, kvinnor i Göteborgs befolkning (från WHO MONICA-studien). Dessa kvinnor återundersöktes 2008 och jämförelser gjordes 2008 med initiala data från 1987 avseende kroppsmått, könshormoner, blodtryck, blodprover, frakturer, livsstilsfaktorer och sjukdomshistoria (via frågeformulär). Bentäthet mättes 1992 och återundersöktes 2008. Via Socialstyrelsens dödsorsaksregister och diagnosregistret erhölls uppgifter om dödsorsak, dödsålder, diabetes, stroke, hjärtkärlsjukdomar och cancer.

Kvinnorna med PCOS (61-79 år gamla) befanns ha fortsatt ökad behåring av manlig typ och högre manlig könshormonhalt i blodet. De hade färre klimakteriesymtom och lägre förekomst av låg ämnesomsättning (hypotyreos) jämfört med kontroller. Den förhöjda midje-höftkvoten hos PCOS-kvinnor före klimakteriet (1987) sågs ej efter klimakteriet. PCOS-kvinnorna ökade i höftomfång, tvärtemot den normala åldrandeprocessen, medan kontrollerna blev mer bukfeta och gick upp i vikt varför skillnaderna i kroppssammansättning mellan kvinnor med PCOS och kontrollerna försvann efter 21 år. Högt blodtryck och förhöjda triglycerider (en viss typ av blodfetter) kvarstod hos PCOS-kvinnor under uppföljningen. Däremot sågs ingen ökad förekomst av frakturer, diabetes, cancer, hjärtinfarkt, stroke eller dödlighet hos kvinnor med PCOS jämfört med kontroller. Kvinnorna med PCOS hade liknande bentäthet som kontrollerna.

Sammanfattningsvis sågs att skillnader i genuttryck föreligger av nyckelproteiner i äggstockarna hos kvinnor med PCOS och kontroller. Detta kan möjligen förklara PCOS-kvinnornas uteblivna ägglossningar samt risken för överstimulering vid hormonstimulering, vid hjälp till graviditet. Kvinnor med PCOS hade lägre förekomst av låg ämnesomsättning, kvarstående högre nivåer av manligt könshormon, ökad behåring, högt blodtryck och höga blodfetter långt efter klimakteriet jämfört med kvinnor i kontrollgrupperna. Trots detta kunde ingen ökad förekomst av frakturer, diabetes, hjärtinfarkt, stroke eller död påvisas under 21 års uppföljning hos kvinnor med PCOS jämfört med kvinnor i befolkningen.

List of papers

This thesis is based on the following papers, which will be referred to by their Roman numerals in the text:

I. **Differential expression of inflammation-related genes in the ovarian stroma and granulosa cells of PCOS women**
Schmidt J, Weijdegård B, Mikkelsen AL, Lindenberg S, Nilsson L, Brännström M
Submitted.

II. **Reproductive hormone levels and anthropometry in postmenopausal women with polycystic ovary syndrome (PCOS): A 21-year follow-up study of women diagnosed with PCOS around 50 years ago and their age-matched controls**
Schmidt J, Brännström M, Landin-Wilhelmsen K, Dahlgren E
J Clin Endocrinol Metab. 2011;96:2178-85.

III. **Cardiovascular disease and risk factors in PCOS women of postmenopausal age: A 21-year controlled follow-up study**
Schmidt J, Landin-Wilhelmsen K, Brännström M, Dahlgren E.
J Clin Endocrinol Metab. 2011. E-pub ahead of print Sept 28.

IV. **Body composition, bone mineral density and fractures in late postmenopausal PCOS women – A long-term follow-up study**
Schmidt J, Dahlgren E, Brännström M, Landin-Wilhelmsen K
Submitted.

Contents

Abbreviations

ACTH	adrenocorticotropic hormone
AES	The Androgen Excess Society
AMH	anti-müllerian hormone
ApoAI	apolipoprotein A-I
ApoB	apolipoprotein B
ASRM	American Society for Reproductive Medicine
BMD	bone mineral density
BMI	body mass index
cAMP	cyclic adenosine monophosphate
CC	clomiphene citrate
CCL2	chemokine ligand 2
cDNA	complementary deoxyribonucleic acid
CI	confidence interval
C_T	cycle threshold
CV	coefficient of variation
CVD	cardiovascular disease
DHEA	dehydroepiandrosterone
DHEAS	dehydroepiandrosterone sulfate
DXA	dual energy X-ray absorptiometry
ESHRE	European Society for Human Reproduction
FAI	free androgen index
FSH	follicle-stimulating hormone
GC	granulosa cell
GnRH	gonadotropin-releasing hormone
HDL	high-density lipoprotein cholesterol
hMG	human menopausal gonadotropin
HOMA	homeostatic model assessment of insulin resistance
IGF-1	insulin growth factor-1
IL1B	interleukin-1 beta
IL8	interleukin-8
IVF	in vitro fertilization
IVM	in vitro maturation
LDL	low-density lipoprotein cholesterol
LH	luteinizing hormone
MI	myocardial infarction
MONICA	MONItoring of trends and determinants for CArdiovascular disease
NOS2	nitric oxide synthase 2
mRNA	messenger ribonucleic acid
NIH	National Institutes of Health
OHSS	ovarian hyperstimulation syndrome
OR	odds ratio
PAI-I	plasminogen activator inhibitor-I
PCO	polycystic ovary
PCOS	polycystic ovary syndrome
pQTC	peripheral quantitative computed tomography
PTGS2	prostaglandin-endoperoxide synthase 2
QPCR	quantitative polymerase chain reaction
rFSH	recombinant follicle-stimulating hormone
RIN	RNA integrity number
SD	standard deviation

SHBG	sex hormone-binding globulin
SPA	single photon absorptiometry
THBS1	thrombospondin 1
TPO	thyroid peroxidase
TSH	thyroid stimulating hormone
T2DM	type 2 diabetes mellitus
T-score	SD of young adults BMD
WHO	World Health Organization
WHR	waist to hip ratio
Z-score	SD of age-matched BMD

Introduction

Polycystic ovary syndrome

The polycystic ovary syndrome (PCOS) is the most common endocrine disorder in women worldwide. The syndrome is characterized by ovulatory dysfunction, hyperandrogenism, and polycystic ovaries (PCO). These features can lead to multiple symptoms with systemic as well as organ-specific aberrations. As PCOS is associated with several other diseases/morbidity-related factors such as obesity and other cardiovascular disease (CVD) risk factors, which are becoming more prevalent among females today, further research on the pathophysiology and the long-term effects of PCOS is of the utmost importance in order to prevent future health problems in the large group of PCOS women.

History

In 1935, Irving Stein (Fig. 1a) and Michael Leventhal (Fig. 1b), both working at the Department of Obstetrics and Gynecology, Michael Reese Hospital, Chicago, USA, described the clinical, the macroscopic characteristics and histological features of PCOS for the first time (1). They had observed an association between amenorrhea, hirsutism and PCO.

Fig. 1a

Fig. 1b

Fig. 1a Photo of Dr. Irving Stein (1887-1976). **Fig. 1b** Photo of Dr. Michael Leventhal (1901-1971). Reprinted with the permission of Simon & Schuster, Inc. from OBSTETRIC AND GYNECOLOGIC MILESTONES- Essays in eponymy by Harold Speert (Macmillan, NY, 1958).

Prevalence

In most studies, the prevalence of PCOS in fertile women is estimated to be between 5-10% (2-4), but the prevalence rates reported are naturally dependent on the exact definition used (5) and on the ethnicity (2, 6) of the studied population. There are no proper studies on the prevalence of PCOS in Sweden, using the modern Rotterdam definition of PCOS. In a large study from Northern Finland an estimated prevalence of PCOS of 3.4% was found, based on response to a postal questionnaire asking a cohort of women born in 1966 about the presence of hirsutism and oligo/amenorrhea (7).

Definitions and phenotypes

The more recent PCOS definitions discussed are the following:

- The NIH criteria were established in 1990 by the National Institutes of Health and included ovulatory dysfunction (amenorrhea or oligomenorrhea) and hyperandrogenism (8).
- The Rotterdam criteria were established in 2003 and revised in 2004 (9) by the European Society for Human Reproduction and Embryology (ESHRE) in collaboration with the American Society for Reproductive Medicine (ASRM). This definition required at least two of the three following criteria: hyperandrogenism, ovulatory dysfunction and/or PCO morphology on ultrasound (see below).
- The AES criteria were set in 2006 by the Androgen Excess and PCOS Society (AES) (10), and they included a requirement of hyperandrogenism in combination with ovarian dysfunction. The latter was defined as ovulatory dysfunction or PCO morphology on ultrasound (see below).

In all these three definitions, hyperandrogenism is defined as clinical and/or biochemical hyperandrogenism. In addition, all these three definitions require the exclusion of other disorders that could mimic PCOS, such as hyperprolactinemia, non-classical congenital adrenal hyperplasia, androgen-secreting tumors and Cushing's syndrome. In this thesis, PCOS is defined according to the Rotterdam criteria, which seem to be the most commonly used criteria today (at least in Europe).

Depending on the PCOS definition used, different phenotypes of the PCOS exist. The division into phenotypes is based on the characteristics of PCOS with oligo/amenorrhea, hyperandrogenism and PCO. The knowledge of the specific phenotypes of a study population is important, as exemplified by the knowledge that there is an increased risk of metabolic dysfunction in women whose phenotype includes hyperandrogenism (10), however most studies have not reported such an increased risk in women with PCO, with or without oligo/anovulation (10).

Morphology of PCO

The definition of the PCO morphology has also varied over the years. The first definition was the one by Stein and Leventhal and they described the macroscopic appearance of PCO ovaries as usually bilateral, enlarged, tense ovaries that were often distinctly globular in shape. The histological description was that of the presence of multiple cysts, rarely larger than 15 mm and these cysts were lined by a hypertrophic theca cell layer. It was also noted that the tunica albuginea, which is the collagen-rich stroma immediately below the ovarian surface epithelium, was much wider than in normal ovaries and that the ovaries were devoid of corpora lutea (1). For comparison, a schematic drawing of the normal ovary is shown in Fig. 2.

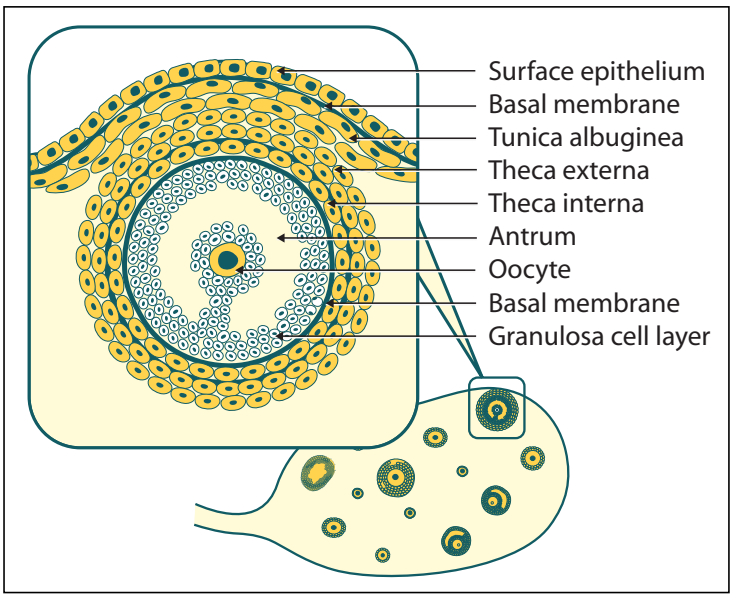


Fig. 2 Schematic drawing of the normal human ovary containing different stages of follicles and corpora lutea. The magnification shows the distinct layers of the follicular wall.

At the introduction of gynecological transvaginal ultrasound in the 1970s and as the ultrasound technology improved, the PCO morphology diagnosis was made using ultrasound instead of ocular inspection or histology. The definition used today is the presence of twelve or more follicles measuring 2-9 mm in diameter, and/or at least one enlarged (>10 cm^3) ovary. If a follicle is >10 mm in diameter, the scan should be repeated (9). The definition does not apply to women taking oral contraceptives. Images of typical ultrasound scan of a normal ovary and of a PCOS ovary are shown in Fig. 3.

The relationship between a PCOS histology and the ultrasound diagnosis has been established (11, 12).

Fig. 3a Fig. 3b

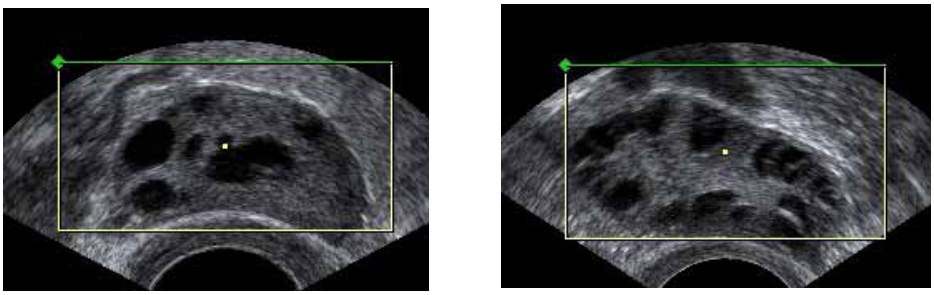


Fig. 3 Typical image by transvaginal ultrasound of a) a normal ovary and b) a PCO ovary. With kind permission of Dr Berit Gull.

Clinical features of PCOS

PCOS is characterized by oligo/amenorrhea, hyperandrogenism and polycystic ovaries. Oligo/amenorrhea is an indicator of oligo/anovulation and is associated with infertility. Oligomenorrhea is usually defined as a menstrual interval of >35 days and amenorrhea is defined as the absence of menstrual bleeding >90 days. Hyperandrogenism is caused by increased ovarian and/or increased adrenal androgen production, which will be discussed further in this thesis. The typical symptoms of hyperandrogenism are hirsutism, acne and/or androgen alopecia; however, the latter being quite a poor marker of androgen excess (9). An illustration of the characteristic clinical features of PCOS and the possible secondary consequences are given in Fig. 4.

The primary clinical indicator of hyperandrogenism is the presence of hirsutism (9), which is a masculine pattern of body hair. The Ferriman-Gallwey system (13) is a scoring system for the extent of hirsutism and seems to be the most widely used system today. The scoring system is based on five grades (with zero being absence of terminal hair) on 11 different body sites: chin, upper lip, chest, upper and lower back, upper and lower abdomen, arm, forearm, thigh and lower leg. However, even if there are many scoring systems, the assessment of the extent of hirsutism is likely to be relatively subjective and a considerable inter-investigator variability has been demonstrated (10). In addition, women have usually treated themselves, before seeking medical attention for their disorder.

Interestingly, Franks found a good correlation between the objective and the subjective grading of hirsutism (11). In 1987, Dahlgren et al. verified these findings in the original study population of paper II-IV (14).

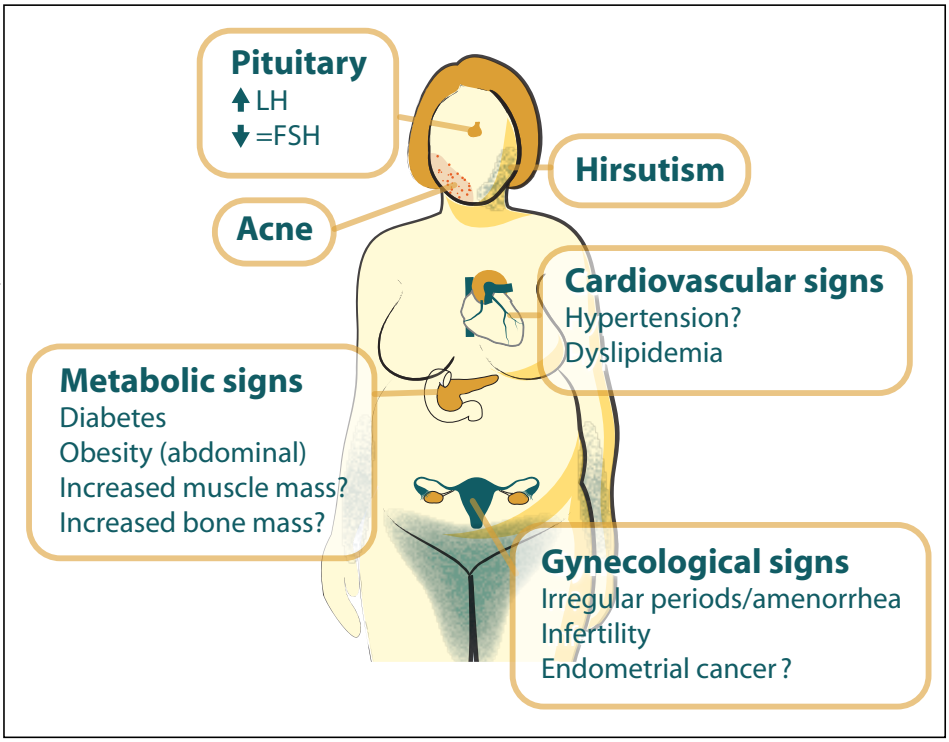


Fig.4 Illustration of the characteristic clinical features of PCOS women of fertile age and the possible long-term consequences. A question mark (?) has been added where no clinical consensus exist.

The usual definition of biochemical hyperandrogenism is a calculated free androgen index (FAI) and calculated values above the 95th percentile of the maximum levels of the reference value applied in the laboratory used for the analysis for the respective androgen sample. The FAI is calculated as the ratio between total testosterone divided by sexual hormone-binding globulin (SHBG) x 100. However, the definition of androgen excess by laboratory measurements is limited, due to the inherent inaccuracy and variability of the laboratory methods used, and due to the fact that there are no well-established normative ranges. Also, age and body mass index (BMI) have not been taken into account when normative values of androgens have been established (9).

The PCOS is also associated with obesity, insulin resistance, diabetes, hyperinsulinemia, hypertension and dyslipidemia (9, 10). There is considerable heterogeneity of the signs and symptoms among women with PCOS and for each woman they may vary over time (10). Weight gain is usually associated with an aggravation of symptoms, while weight loss usually ameliorates the symptoms and the endocrine and metabolic disturbances (15, 16). Interestingly, an effective weight loss of only around 5% can reverse the PCOS-associated anovulation (15).
Depending on the criteria used, the prevalence of the classical clinical features of PCOS varies with approximately 66-75% with menstrual dysfunction (2, 17, 18), 60-69% with hirsutism/acne (2, 17, 18), 48-80% with increased androgens levels (2, 18), and in patients defined by the AES criteria ~75% had PCO morphology (10).
There is a lack of data regarding clinical features in postmenopausal PCOS women, which is one of the main foci of the present thesis.

Etiology and pathophysiology of PCOS

The pathogenesis of PCOS is multifactorial and far from completely understood. Multiple causative mechanisms are discussed, involving interactions between certain genes and environmental factors (6, 19), dysfunction/regulation by the gonadotropins and intra-ovarian factors, hyperinsulinemia as well as hyperandrogenism. An illustration of the proposed pathophysiological characteristics of the PCOS is given in Fig. 5.

Genetics

There is evidence of a genetic component based on the existence of familial clustering (20-22) and twin studies have displayed a two fold increased concordance of PCOS in genetically identical twins compared with non-identical twins (23). In spite of numerous association studies (mainly focusing on genes associated with the synthesis and metabolism of androgens and insulin), the way in which PCOS is inherited remains unclear (24). Recent efforts, using modern mapping techniques, have made some progress to identify promising candidate genes. Two promising candidate genes have so far emerged. The first, a locus on chromosome 19p13.2, is associated with high susceptibility to PCOS (25) and the second is the fat-mass and obesity associated gene, whose polymorfism has have been found to be associated with PCOS (26). However, the studies implicating these two locus, needs to be confirmed in larger studies and in other populations.

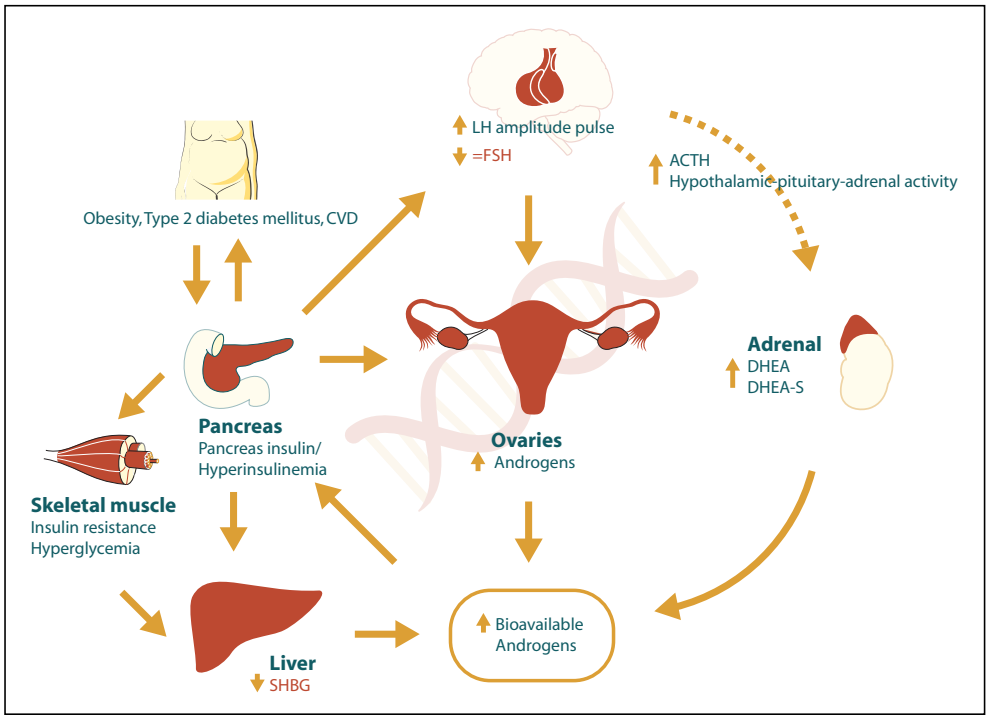


Fig. 5 Simplified illustration of some of the different organ-specific aberrations and their interactions in the pathophysiology of PCOS. Gonadotropin aberrations: The increased frequency of LH pulses from the pituitary gland is secondary to increased frequency of GnRH pulses in the hypothalamus. This leads to increased pituitary production of LH.
Ovarian aberrations: The elevated levels of LH lead to increased androgen production from the theca cells. The relatively lower FSH levels contribute to arrested follicular development in the ovary, which, in turn leads to disturbed negative feedback. This results in continued aberrations in the secretion of LH and FSH.
Aberrations in the adrenal gland: Impaired adrenal androgen production leads to increased levels of DHEA and DHEAS, which, in turn, also increases the circulating pool of free and bioavailable androgens.
Pancreatic aberrations: The increased levels of bioavailable androgens lead to increased insulin resistance in peripheral tissues (mostly in the skeletal muscle). This leads to hyperinsulinemia, which, by facilitating the stimulatory role of LH, leads to increased ovarian androgen production. Moreover, increased release of free fatty acids from adipocytes is seen, due to insulin resistance and hyperandrogenism (27).
Liver aberrations: Insulin-induced decreased production of SHBG leads to an increased amount of free androgens.
Peripheral tissue: The insulin resistance and the hyperinsulinemia could cause obesity and T2DM, which increases the CVD risk and could lead to CVD. Genetic factors: All the aberrations mentioned could, in concert with genetic factors, lead to the PCOS and eventually in an adverse CVD risk profile.
ACTH=adrenocorticotropic hormone, CVD=cardiovascular disease, DHEA=dehydroepiandrosterone, DHEAS=dehydroepiandrosterone sulfate, FSH=follicle-stimulating hormone, GnRH=gonadotropin-releasing hormone, LH=luteinizing hormone, SHBG=sexual hormone-binding globulin.

Environmental factors

Regarding the origin of PCOS, environmental factors such as prenatal exposure to androgens and weight gain have been discussed as contributing factors; thus, it may well be that genetic factors give a high susceptibility to PCOS and that the syndrome will develop only in the added presence of a specific environment, most likely with exposure during fetal life or early childhood.

Prenatal exposure to androgens

Excess fetal exposure to maternal androgens is thought to contribute to inducing the PCOS phenotype in offspring/children, based on experimental data from animal studies as well as clinical material of pathological conditions in human populations (i.e., congenital adrenal hyperplasia) (28, 29). In humans, higher testosterone levels, which were elevated to male levels, have been found in the umbilical vein in female infants born to mothers with PCOS (30). However, the only prospective study of the relationship between prenatal androgen exposure and the development of PCOS during the human female adolescence did not confirm any association between these variables (29).

Obesity

Obesity has a considerable effect on the manifestation of PCOS (31) and family studies have implied that weight gain may promote the PCOS phenotype in a predisposed population (32). Weight gain is usually associated with a worsening of symptoms, while weight loss usually ameliorates the symptoms and the endocrine/reproductive and metabolic disturbances (15, 16). The prevalence of obesity/overweight varies in different countries and a study in the US has shown that 42% of the PCOS population were obese (BMI >30 kg/m²) and 24% were overweight (BMI 25-29.9 kg/m²) (2). Studies in Europe have shown a thinner PCOS population with mean BMIs in the UK, Greece, Finland and the Netherlands in the range of 25-29 kg/m² (3, 4, 7, 33). On average, 10-40% of PCOS women are known to be obese (BMI>30 kg/m²) (3, 4) and 40-90% have been shown to be overweight (BMI>25 kg/m²) (34).

Hypothalamus/pituitary - ovarian axis dysfunction

A large proportion of women with PCOS have increased levels of LH (35, 36) and normal/decreased levels of FSH (37, 38), resulting in the discussed classical hormonal hallmark of an increased LH/FSH ratio. The prevalence of an increased LH/FSH ratio is partly related to BMI, and it is more prevalent in PCOS of normal weight and less common with increasing BMI (39). The increase in LH is explained by an increased pulse frequency of the hypothalamic gonadotropin-releasing hormone (GnRH) (36), which

may favour the production of the β-subunit of LH over the β-subunit of FSH (40), and/or by increased pituitary sensitivity to GnRH stimulation (41). The increase in LH causes the ovaries to favour the production of androgens from the theca cells carrying LH receptors.

Partly due to the increased LH stimulation there is increased ovarian production of androgens mainly from the theca cells. The theca cell layer in follicles of PCO has been shown to be thicker (42) and androgen hypersecretion and increased expression/efficacy of the key enzymes participating in the synthesis of androgens has been verified (43, 44). The follicular steroid secretion follows the two-cell cooperation, where LH-stimulated theca cells produce mainly androstenedione from the steroid precursor cholesterol and via pregnenolone, progesterone and 17-OH-progesterone. Androstenedione is converted to testosterone after diffusion through the basal lamina to the granulosa cells (GC). This cell compartment is rich in aromatase and consequently androstenedione is aromatized to estrone or testosterone. Testosterone is aromatized to estradiol (42, 45), see Fig. 6. The androgens, and in particular androstenedione, is taken up by diffusion to the capillaries of the theca and may then undergo aromatization in skin, liver and adipose tissue to estradiol after conversion to testosterone (46). In addition, insulin increases the response of the theca cells to LH, resulting in increased androgen production (47, 48), and hyperinsulinemia is common in women with PCOS (49).

In the GCs, FSH stimulates the expression of enzymes that metabolize androstenedione to estradiol (45). Studies of follicular fluids and in vitro studies of GCs from anovulatory PCOS women demonstrate that GCs, for the most part, remain steroidogenically active with increased aromatase activity, compared with similarly sized follicles from non-PCOS women. Thus, increased estradiol production in PCOS is dependent on the ovulatory status of the patient (50), but also on body weight (11). Consequently, also normal estradiol levels have been found in PCOS (11, 51).

Anti-müllerian hormone (AMH) is a specific hormone of small growing follicles being produced in GCs of primary follicles and the growing follicles continue to express AMH until the time they are selected for dominance by FSH (52). After the selection of the dominant follicle, the GCs normally start to produce inhibins and estradiol that cause a progressive decline in FSH by negative feed-back (53). In PCOS, the primary follicle pool is much higher than in normal women and the number of antral follicles, as assessed by ultrasound, is shown to correlate tightly with the serum AMH levels, which also has been found to be 2-3 times higher than in non-PCOS women (54, 55). The increased AMH

levels could be one factor that influences follicular maturation (54). The high androgen levels and this AMH-related mechanism may be factors behind the follicular arrest, which is the basis of the characteristic appearance of PCO with arrested multiple small follicles <10 mm in diameter.

Concerning androgens and follicular arrest, there is a positive correlation between the number of arrested follicles and androgen levels (6). These high androgen levels and the excessive stimulation of follicular cells by insulin and LH might produce high levels of cyclic adenosine monophosphate in the GCs, which may result in premature terminal differentiation and, hence, arrest follicular growth (56).

Taken together, it is likely that the abnormal endocrine environment in PCOS women, with the hypersecretion of LH, androgens and insulin, together with the relative FSH deficiency (57, 58) and increased AMH levels, impair the development of the maturing pool of follicles (54).

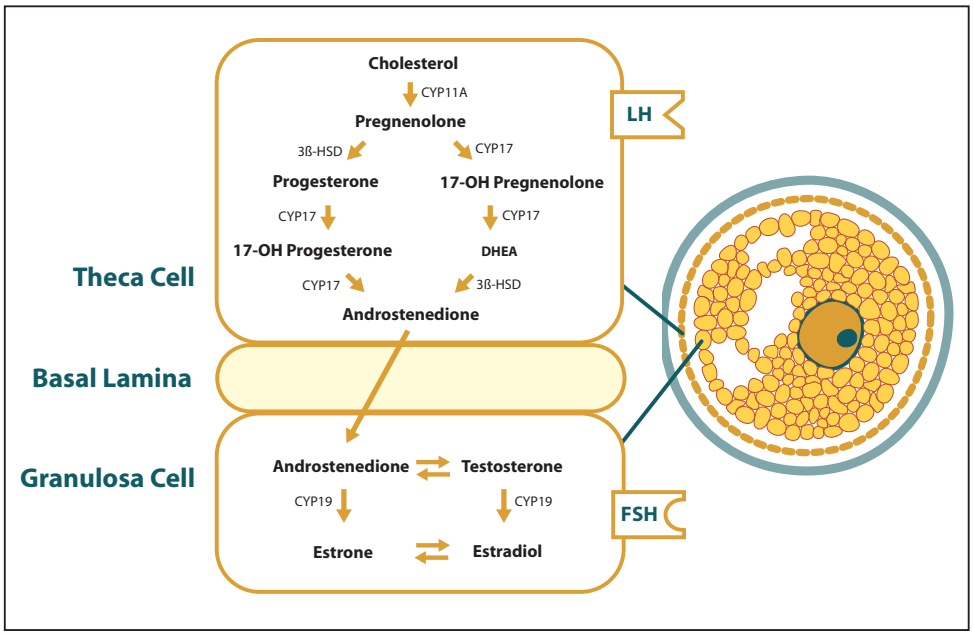


Fig. 6 The steroidogenesis in the theca and granulosa cells of the ovary. LH stimulates the theca cells after receptor binding, which, by second messenger activation involving cyclic adenosine monophosphate, leads to increased expression of cholesterol side chain cleavage cytochrome P450 (CYP11A), 17αhydroxylase/C17,20 lyase cytochrome P450 (CYP17), and 3β-hydroxysteroid dehydrogenase (3β-HSD). The theca cell is then able to synthesize androstenedione from cholesterol. Androstenedione diffuses across the basal lamina into the granulosa cells and in normal ovaries, the major part of androstenedione is converted into estrone by aromatase cytochrome P450 (CYP19) and then to estradiol by 17 β –hydroxysteroid dehydrogenase (17β-HSD). However, in PCOS ovaries, testosterone is produced (by conversion by 17β-HSD) to a larger degree from androstenedione.

Adrenal androgen production

The adrenal cortex synthesizes all the three major androgens; dehydroepiandrosterone sulfate (DHEAS), androstenedione and testosterone, and this is the other major site of female androgen production, besides the ovaries. DHEAS is almost exclusively (97-99%) produced by the adrenal cortex and androstenedione is produced in both the adrenal gland and the ovaries (46), whereas 25% of testosterone is synthesized by the adrenal gland, 25% in the ovary and the remaining part being produced through peripheral conversion from androstenedione in liver, adipose tissue and skin (46). Around 60-80% of PCOS women have high concentrations of circulating testosterone (59).

In PCOS women, the prevalence of DHEAS excess is 20-30%, depending on ethnicity and DHEAS levels decline up to the age of ~ 45 years (60). The increased DHEAS levels in PCOS women compared with controls is verified up to the perimenopausal ages (61). However, the mechanisms of the adrenal androgen excess in PCOS is still unclear, although it has been proposed that it may result from increased metabolism of cortisol, which could lead to decreased negative feedback on ACTH secretion (62).

SHBG production

SHBG is produced in the liver. Women with PCOS have decreased levels of SHBG, which is caused by inhibitory effects of insulin on the SHBG production (63, 64). In addition, overweight/obesity decreases SHBG production even more (65). Decreasing SHBG levels result in increased levels of biologically active androgens, as normally about 80% of testosterone (64) and 8% of androstenedione (66) is generally bound by SHBG, with the other main binding protein being the constitutively expressed albumin (64).

Insulin resistance

Insulin resistance, i.e., impaired stimulation of glycogen formation in all major target tissues (skeletal muscle, adipose tissue, liver, kidney), is a pathogenic characteristic feature of PCOS, particularly among obese subjects (67). The molecular mechanisms of insulin resistance involve defects in the insulin-receptor signalling pathway in both adipocytes and in skeletal muscle (68).

Insulin resistance causes compensatory hyperinsulinemia and might contribute to hyperandrogenism and gonadotropin aberrations through several mechanisms. Insulin may act directly in the hypothalamus, the pituitary or both and thereby contribute to abnormal gonadotropin levels (69). High insulin can also serve as a co-factor to stimulate

ACTH-mediated androgen production in the adrenal glands (70). As stated above, the stimulation of the ovaries is exerted by a synergistic effect of insulin upon LH stimulation of the theca cells (47, 48) and insulin may also directly stimulate theca cell proliferation (49). In addition, high insulin concentrations also cause decreased circulating SHBG, thereby increasing the levels of free bioavailable testosterone (63, 71). Administration of insulin in young non-PCOS women resulted in increased LH-puls frequency, thereby implying an association between insulin and hypothalamus-pituitary-ovarian-axis-activity (72).
Hyperinsulinemia also results in increased levels of free IGF-1 and human theca cells express IGF-1 receptor genes (as well as insulin receptor genes), which is another way in which androgen production is stimulated (73). In addition, free IGF-1 is a potent growth factor that can induce proliferation of ovarian cells (74).
In conclusion, excess of androgens in PCOS of ovarian and/or adrenal origin initiates or maintains a vicious circle, where hyperandrogenism leads to hypothalamus/pituitary abnormalities, ovarian dysfunction, insulin resistance and abdominal obesity, which in turn stimulates further androgen production (75).

Summary of endocrine disturbances in PCOS women of fertile age

In summary, the characteristic endocrine picture of PCOS women of fertile age is that of increased LH (35, 36), normal to low FSH (37, 38), increased androgens (11, 60), normal to low SHBG (38) and normal to increased estradiol levels (11, 51).

Consequences of PCOS

Reproductive consequences of PCOS

Infertility

Among PCOS women around three quarters are subfertile or infertile (17), which is mostly due to oligo/anovulation and metabolic alterations (76). First-line treatment of infertility associated with PCOS are weight reduction (77) (if overweight) and/or clomiphene citrate (CC), taken orally. However, approximately 20% of PCOS women treated with CC are so called “CC-resistant” (78), usually defined as failure to ovulate on a dose up to 150 mg CC for five days or, if ovulating, failure to conceive within 3 months. For these women, an addition of the insulin-sensitizing drug metformin to CC therapy has been discussed and beneficial effects have been seen, mainly in small single-center trials that have mainly focused on metabolic and hormonal measures, rates of ovulation or both,

rather than on live birth rates (79). However, a large (n=626) randomized study showed no improved live birth rate with a combination of CC treatment and metformin compared with CC treatment alone (live-birth rate 26.8% compared to 22.5%, ns) (80), althought, multiple birth was a complication (80).
Second-line treatment to induce ovulation in PCOS is low-dose gonadotropin therapy (81), using human menopausal gonadotropins (hMG) or recombinant FSH (rFSH). Gonadotropin therapy is widely used but carries a risk of multiple pregnancies and hyperstimulation syndrome (OHSS) (82).

An alternative to low-dose gonadotropin therapy for achieving pregnancy in anovulatory PCOS is surgery. The first to report on this procedure were Stein and Leventhal in 1935 (1). They described ovarian wedge resection in seven women. The surgery resulted in regular periods in all seven women and two pregnancies in two of the formerly infertile patients during four years. This procedure was abandoned due to postoperative adhesions, the introduction of CC therapy and the development of laparoscopic surgery.
The first type of laparoscopic surgery for PCOS women with infertility was described and introduced by Gjonnaess in 1984 (83). Gjonnaess applied unipolar electrocautry to the ovarian capsule for 2-4 seconds until the capsule ruptured. This technique, called laparoscopic ovarian drilling, has been altered by many researchers since then, but Gjonnaess´s technique largely remains the predominant procedure. However, a study in 2005 showed that 5 instead of 10 punctures per ovary was enough to maintain the same ovulation and pregnancy rate and the amelioration of the hyperandrogenic status (84).
The aim of all the different techniques of ovarian wedge resection/ovarian drilling is to create endocrine reversal, and from an endocrine perspective the different techniques can be considered as equivalent (85). However, the mechanism of action behind the endocrine reversal is still not fully understood, even if reduction/destruction of large parts of the ovarian androgen-producing tissue is a likely primary mechanism, with secondary normalization of the feed back systems between the ovary and the pituitary. With restoration of ovulation after ovarian drilling, the serum concentration of testosterone and LH falls. Whether women respond to ovarian drilling or not seems to be dependent on pre-treatment characteristics; women with high basal LH seem to have a better clinical and endocrine response (86).
Comparing gonadotropin therapy and ovarian drilling, smaller studies have indicated that the cumulative conception rates are approximately similar (87) and after 6 months of treatment the conception rate ranged from 38-62% (87, 88).

If pregnancy is not achieved by any of the treatments described above, the method of choice is in vitro fertilization (IVF). IVF is performed by controlled ovarian hyperstimulation with higher doses of rFSH or hMG (compared with ovulation induction with gonadotropin therapy), with the goal (at the moment in Sweden) of achieving ~10 matured follicles (during the same menstrual cycle). The follicles are then transvaginally punctured to aspirate the follicular fluids and the cumulus-enclosed oocytes for further separation of the oocytes for IVF. If an embryo, considered to be of good enough quality, has developed, the embryo (in the 4-8 cell or blastocyst stage) is transferred *in uteri*, usually two to three or (if transfer of a blastocyst) five to six days after the aspiration. Compared to infertility caused by other factors, the PCOS women have a better chance of having more follicles that could be aspirated after stimulation, but women with PCOS, on the other hand, run the greatest risk of developing ovarian hyperstimulation syndrome (OHSS), a risk which is considered to be ~15% for PCOS women (for severe OHSS) compared to ~3% for women with normal ovaries (89). The described risk of OHSS varies with the PCOS definition and the OHSS definitions used.

Towards the end of the 1990s, *in vitro* maturation (IVM) was developed, mainly with the purpose of making IVF safer and simpler for women with PCOS. The advantage of IVM compared with IVF is the avoidance of OHSS, which is especially beneficial for women with PCO/PCOS. Furthermore, the treatment is easier for the patients with lower doses of medication and thereby lower costs and shorter treatment time. The difference between IVM and IVF is that the oocytes undergo maturation *in vitro* instead of *in vivo*. Hence, the goal is to obtain follicles of a size between 2 and 10 mm. The oocytes of these follicles will be arrested in meiosis and are by their morphological appearance, with a distinct nuclear membrane, referred to as oocytes of the germinal vesicle stage. They will then undergo germinal vesicle breakdown and expulsion of polar bodies *in vitro* to become haploid and mature metaphase II oocytes, which can then be fertilized.

IVM has been reported for two main groups of women. The first group is regularly cycling women with normal ovaries and the second group is women with PCO/PCOS.

In women with normal ovaries (normal follicular distribution), IVM could be achieved without any stimulation at all. In PCOS women, rFSH or hMG is usually given, but only for three days to achieve pregnancy and implantation rates at the same levels as when performing IVM in non-PCOS women (90). The avoidance of human chorionic gonadotropin and the low or zero doses of rFSH/hMG make the risk of OHSS extremely low (90). A higher cancellation rate of IVM cycles is reported compared with IVF, the aspiration process is more difficult and the success rate is lower than after IVF (91), which is why the IVM technique, so far, is not widely used.

Ovarian reserve

The ovarian reserve represents the pool of small follicles within the ovarian cortex that can develop into larger follicles with oocytes that are capable of fertilization. Different variables, such as measurement of FSH, the number of ultrasound detected antral follicles, and inhibin B and AMH levels (92), together with the patient's menstrual cycle length, have been used to estimate the ovarian reserve. Measurement of the ovarian reserve is used in reproductive medicine to try to estimate the outcome of stimulation and thereby getting an idea about the most appropriate starting dose for the specific patient. This is specifically important in PCOS women, as too high a dose of rFSH or hMG increases the risk of OHSS.

The blood levels of FSH are highly variable during the menstrual cycle and in women of fertile age below ~40 years of age, the results regarding differences in FSH levels between PCOS and non-PCOS women have been inconclusive (57).

The exclusively ovarian derived hormone AMH is expressed exclusively in the GCs of the growing follicle from the primary stage in the ovary up until the antral stage and until the dominant follicle is selected (52) and the levels correlate well with the number of antral follicles, as assessed by ultrasound (54, 55, 93). In addition, levels are virtually unchanged during the menstrual cycle (94) and AMH is therefore the best marker of the ovarian reserve, so far. The AMH levels in PCOS women, are two to three-fold higher than in controls in several studies (54, 95) and the number of antral follicles are ~6 times higher in anovulatory PCOS women compared with normal ovulatory women (96). Ovaries of PCOS women are generally larger than the ovaries in non-PCOS women. Taken together, these observations suggest that PCOS women, in general, have a larger ovarian reserve.

Through reproductive life of a woman, the AMH levels gradually decrease until no levels can be detectable around five years before the final menstrual period (97). An opposite age-dependent pattern is described regarding FSH, which increases during reproductive life, making it possible to use FSH as a marker of the perimenopause/menopause.

Pregnancy complications

Miscarriage rates are believed to be higher in PCOS women than in normal women, although it is discussed whether it is the PCOS *per se* or the associated overweight/obesity that is the actual cause. A recent meta-analysis verified the results of several other studies and showed an increased prevalence in PCOS women of gestational diabetes, gestational hypertension, preeclampsia and premature births. In addition, the infants of PCOS women were more often admitted to a neonatal intensive care unit and the perinatal mortality was higher, independently of multiple pregnancies/deliveries (98) .

Muscle, bone and PCOS

Osteoporosis is defined as "a disease characterized by low bone mass and microarchitectural deterioration of bone tissue, leading to enhanced bone fragility and a consequent increase in fracture risk" (99). In general, 30% of all women aged over 50 years will develop osteoporosis (100), which is defined by the WHO criteria as bone mineral density (BMD) ≤-2.5 standard deviations (SD) below that of a young adult (T-score) (101).

The methods for examining BMD have changed over the last decade due to the development of more sophisticated techniques that can determine BMD more accurately. In the early 1980s, the dominant technique for bone densitometry was single photon absorptiometry (SPA), generally applied to the distal non-dominant forearm (102). During the 1990s Dual-energy X-ray Absorptiometry (DXA) was introduced and, later, also peripheral Quantitative Computed Tomography (pQTC). The DXA method evaluates bone mass, which is an indirect factor when estimating bone strength and, in addition, body composition can also be assessed (103). This method is referred to as the "gold standard" for assessing BMD in clinical practice. PQTC enables quantification of the trabecular and cortical densities, bone density and volumetric bone density. It has been discussed whether this technique might be superior to DXA as pQTC also provides information on bone strength. However, a major disadvantage of pQTC is the high dose of radiation; nor has this technique been shown to be superior to DXA at predicting fragility fractures (104).

Numerous studies and one meta-analysis have shown that measurements of BMD can predict the fracture risk; on the other hand, it cannot identify individuals who will later have a fracture (105). The clinical significance of osteoporosis lies in the fractures that occur and the typical major osteoporotic fractures are vertebral, hip and distal forearm fractures. Risk factors for osteoporosis and, thus, for fractures are age, low BMI, smoking, physical inactivity, low calcium and vitamin D intake, excessive daily alcohol consumption, long-term use of oral glucocorticoids and certain diseases, such as rheumatoid arthritis (106). Any osteoporotic fracture may have severe consequences for the individual and are a burden to the health care system and to society.

Androgens are positively associated with muscle mass and BMD (107-111). It has been speculated whether PCOS women actually have increased muscle mass and/or BMD. This could hypothetically lead to a lower incidence of fractures. It is of interest to study these aspects, especially in postmenopausal PCOS women who have reached the age when a majority of fractures occur.

Speculations also exist whether amenorrhoic PCOS women would have a lower BMD than regularly cycling PCOS women, based on the fact that amenorrhoic women, in general, are known to have an increased risk of osteoporosis and fractures (100). However, a large retrospective study of women with PCOS morphology and amenorrhea showed higher BMD in amenorrhoic PCOS compared to amenorrhoic non-PCOS (112).

Studies on BMD and lean mass in PCOS women are difficult to compare properly, due to the use of different inclusion criteria for the PCOS groups, different ages, different BMIs of the study populations, and lack of controls (113). Also, the PCOS and control groups compared sometimes have different age and/or BMI (114, 115). An overview of studies examining on BMD and/or lean mass in PCOS women is shown in Table 1.

Table 1 Studies examining BMD and/or muscle mass in women with PCOS

Study	PCOS definition	Patients (n)	Mean age (years)	Mean BMI (kg/m^2)	Matching	Method	Results in PCOS
Adami S et al., 1998 (114)	HA and anovulation with/without PCO	P: 51 C: 35	P: 24.2±4.9* C: 29±6	P: 23.5±4.8 C: 21.7±3.2	no	DXA	BMD=
Good C et al., 1999 (116)	HA and amenorrhea	P: 12 C: 10	P: 28.5±7.0 C: 28.9±8.3	P: 22.4±2.3 C: 22.2±2.0	age, weight, ethnicity	DXA	BMD in upper extremities ↑ BMD=other sites
Kirchengast S et al., 2001 (117)	HA and oligo/ amenorrhea and PCOS	P: 10 C: 10	P: 23.9 (18-39) C: 22.9 (19-29)	P: 20.7±1.4 C: 20.4±1.6	age, weight, BMI	DXA	BMD= lean mass ↓
To W. et al., 2005 (115)	Oligo/ amenorrhea and PCO	P: 14 C: 45	P: 16.6±1.6 C16.9±1.4	P: 23.4±4.6* C: 20.8±4.1	age	DXA	BMD=
Carmina E et al., 2009 (118)	HA and chronic anovulation and/or PCO	P: 95 C: 90	P: 24.2±3 C: 23.9±3	P: 27.6±5.8 C: 26.8±3	age, weight,	DXA	BMD= lean mass ↑
Kassanos D et al., 2010 (103)	HA and oligo/ anovulation and/or PCO	P: 30# C: 15	P:L 26.5±3.68 P:O 28.5±4.08 C: 26.7±4.4	P: L 22.3±3.3 P: O32.3±2.9*[a] C: 23.6±3.2	age	pQTC	Volumetric cortical density ↑

All studies above have a cross-sectional design. All but the study by Kassanos et al. were performed using DXA. BMD=bone mineral density, DXA=dual energy X-ray absorptiometry, pQTC=peripheral computed tomography. HA=hyperandrogenism. P=PCOS women, C=controls. Equal sign (=) has been added where BMD or lean mass was similar in PCOS women and controls. ↑ has been added where BMD or lean mass was higher ($p<0.05$) in the PCOS women than in the controls. ↓ has been added when BMD or lean mass was lower ($p<0.05$) in PCOS women compared with controls.

#) 15 Lean, 15 Obese PCOS patients. PCO=PCO morphology on ultrasound, L=lean, O=obese, P=PCOS, C=controls, *= $p< 0.05$, *[a]) lean PCOS BMI ns compared with BMI of controls.

The postmenopausal PCOS women have most likely been exposed to their higher levels of androgens for decades. However, there are no studies of BMD, lean mass or fractures in perimenopausal or postmenopausal PCOS women.

Cancer and PCOS

Endometrial, ovarian and breast cancer are mainly discussed as having a possible association with PCOS.

Risk factors for endometrial cancer are excessive weight, hyperinsulinemia, nulliparity, and a longer time of exposure of estrogens (119), and these factors are also associated with PCOS. In addition, increasing age and a sedentary lifestyle add to the already mentioned risk factors (119). The mechanism behind endometrial cancer in PCOS that has been discussed is the unopposed stimulation by estrogens of the endometrium, which can cause endometrial hyperplasia with increased risk of atypia and eventually endometrial cancer (119). No large prospective studies regarding PCOS and endometrial cancer exist and the results of studies are conflicting. However, a recent meta-analysis showed an almost three times higher risk of developing endometrial cancer for PCOS women (OR 2.70;95% CI 1.00-7.29) compared to women without PCOS (120).

Regarding breast cancer, the discussed cause of an association with PCOS, is that of obesity, hyperandrogenism, the longer time-periods of unopposed estrogen and of infertility (121). Most studies of PCOS and breast cancer show no increased risk for women with PCOS, as supported by two review articles on the subject (120, 121) and the meta-analysis by Chittenden et al. (120).

The risk of ovarian cancer seems to be increased in women with multiple ovulations i.e., nulliparity, late menopause and early menarche (121). Many of these factors are present in PCOS women and, theoretically, these women could have an increased risk of ovarian cancer, although this is contradicted by the fact that a large percent of women with PCOS are oligo/anovulatoric.

As for endometrial cancer and breast cancer, data of an association between PCOS and ovarian cancer are conflicting, studies are small and prospective studies are lacking. Most studies seem to show no association between ovarian cancer and PCOS (121, 122), but one small population-based case control study reported a ~2-fold increased risk (123).

The studies of cancer and a possible association with PCOS have been performed on women below the age of 50 years. The risk of the cancer types mentioned above increases with age, as most of the cases of endometrial cancer are diagnosed after the menopause, with the highest incidence around the age of 70 years (119). It is important to investigate postmenopausal women with PCOS regarding these issues.

Metabolic Consequences of PCOS

Insulin resistance and type 2 diabetes mellitus (T2DM)

The diagnosis of insulin resistance can be confirmed in several ways. The euglucemic-hyperinsulinemic glucose clamp is the golden standard, but a cumbersome method. The easiest method is based on results of calculations based on peripheral basal fasting insulin and glucose, such as the homeostatic model assessment of insulin resistance (HOMA) (124).

Insulin resistance is strongly associated with PCOS and causes compensatory hyperinsulinemia. Most women with PCOS are able to compensate fully for their insulin resistance. However, as a substantial proportion of PCOS women have disordered and insufficient beta-cell response (125), causing glucose intolerance, they are at a high risk of developing T2DM.

Around 50-70% of PCOS women have been found to be insulin-resistant and hyperinsulinemic (47), and PCOS women have an increased risk of impaired glucose tolerance and T2DM at all body weights and even at young ages according to Legro et al (126). However, there are conflicting data on insulin sensitivity in lean PCOS women compared with BMI-matched and weight-matched controls, with a few studies indicating similar prevalence (127, 128), some demonstrating reduced insulin sensitivity (67, 126, 129), and some stating that only hyperandrogenic PCOS women develop insulin resistance (130, 131). The different results may be explained by the diversity of the used criteria for the PCOS diagnosis and the heterogeneity of the PCOS population. Nevertheless, the insulin resistance of PCOS women is closely connected with especially hyperandrogenism and abdominal obesity (75, 132-134) and at a lower BMI than in healthy women (68, 135).

A study in the US showed that ~30% of obese PCOS women <45 years have impaired glucose tolerance after oral glucose tolerance test, but lean (<27 kg/m²) PCOS women have a lower frequency of ~10%. Of the obese subjects, 7.5% had diabetes compared with 1.5% in the lean group (126). In a study of Mediterranean women with PCOS (mean age <28 years) Gambineri et al showed ~16% glucose intolerance and 2.5% T2DM (136).

The metabolic syndrome

The metabolic syndrome has many definitions. According to the American Heart Association and the US National Cholesterol Education Program/Adult treatment panel III (NCEP/ATPIII), the metabolic syndrome is defined by ≥3 of the following metabolic abnormalities: waist circumference (abdominal obesity) >88 cm, fasting serum

triglycerides ≥1.7 mmol/L or on drug treatment for elevated triglycerides, high-density lipoprotein cholesterol (HDL) <1.3 mmol/L, blood pressure ≥130/≥85 mmHg or on antihypertensive drug treatment in a patient with a history of hypertension and/or fasting serum glucose ≥6.1 mmol/L or on drug treatment for elevated glucose (137). PCOS has many features in common with the metabolic syndrome (138) and the prevalence varies between 1.6% to 46% (138-140) in PCOS women and is highly associated with age and body weight (138). In the general female population the metabolic syndrome is associated with an increased risk of diabetes mellitus, CVD (141) and all-cause mortality (142).

Obesity/abdominal obesity

Abdominal obesity is the metabolically most detrimental type of obesity and has been shown to be associated with insulin resistance and T2DM, hypertension, dyslipidemia and CVD (143-147). Increased BMI and abdominal obesity is also associated with increased levels of fibrinogen and PAI-1 (148) and an increase in inflammatory markers (147). Several studies show that PCOS women of fertile age have increased abdominal obesity with increased visceral/abdominal fat (117, 133) compared with normal controls. The methods for measuring abdominal fat have been discussed for several years and DXA, magnetic resonance imaging (MRI) and computer tomography (CT) have been used. However, abdominal obesity, measured as waist to hip ratio (WHR), has recently been shown to be a strong risk factor for myocardial infarction (MI) (and to be stronger related than BMI) and has been recommended for use in the assessment of CVD risk (143) in a large study including populations in 52 countries (the INTERHEART study). In addition, both waist and hip circumference are two easily used, available, cheap and patient-friendly measurements.

A WHR >0.85 or a waist circumference >88 cm are both correlated to hyperandrogenism, increased glucose intolerance, dyslipidemia and an consequential increased risk of CVD (137).

Hyperlipidemia

Elevated serum lipids are well known risk factors for CVD, while HDL may have a protective effect (149). According to the National Cholesterol Education Program (NCEP), ~70% of PCOS women have abnormal serum lipid levels (146), and dyslipidemia might be the most common metabolic abnormality in PCOS (27). The dyslipidemia in PCOS includes elevated levels of LDL and triglycerides and decreased levels of HDL (27, 150-152).

Recent studies have shown low Apo A1, high ApoB and a high Apo B/Apo A1 ratio to be superior predictors of acute MI in both sexes and at all ages (143, 153, 154). Apo A1 is

the major protein component of HDL and promotes cholesterol efflux from the liver. Apo B, a lipoprotein of LDL, is a ligand for LDL receptors and facilitates the LDL cholesterol distribution to tissues. In PCOS, studies on Apo levels have included only young women and the results indicate lower Apo A1 and similar Apo B as in non-PCOS women (150).
As PCOS women tend to have increased abdominal fat, they are more predisposed to dyslipidemia, as the centrally located adipocytes seem to exert an adverse effect on blood lipids (155). Within the adipocytes in PCOS, insulin resistance and hyperandrogenemia, result in increased catecholamine-induced lipolysis and the release of free fatty acids into the circulation. Elevated free fatty acids flux to the liver and stimulate the assembly and secretion of very low-density lipoprotein producing hypertriglyceridemia (27). Centrally located fat is more insulin-resistant and recycles fatty acids more rapidly through lipolysis compared with peripheral fat (27, 156, 157) .

Hypertension

Hypertension is the fourth strongest modifiable risk factor for MI (143) and one of the strongest risk factors for stroke (158). In PCOS women, studies on blood pressure have given inconsistent results. Relatively mild increased blood pressure was seen in some studies of premenopausal PCOS women (138, 159, 160), and some reported normal mean blood pressures (152, 161, 162). The diversity of results may depend on incongruence of age of the study populations, as hypertension develops late due to insulin resistance and as hypertension has been verified in perimenopausal PCOS (61).

Cardiovascular consequences of PCOS

CVD risk factors

CVD is estimated to be the major cause of death worldwide (163). Several risk factors for CVD/MI exist and they can be grouped into non-modifiable and modifiable factors.
The major non-modifiable CVD risk factors in women are age and family history of premature CVD. Modifiable risk factors for CVD/MI are high cholesterol, diabetes, hypertension, abdominal obesity, smoking, alcohol, physical inactivity and psychosocial stress, and these explain more than 90% of the risk of acute MI (143, 164). Similar odds ratios were found regarding all the risk factors in both sexes, except that the increased risk associated with hypertension and diabetes, and the protective effect of exercise, seemed to be greater in women than in men (143). Multiple studies show an increased frequency of CVD risk factors in PCOS women of fertile/premenopausal ages. It has also been shown that mothers of women with PCOS had a higher risk of CVD events than controls (165) and, in addition, that PCOS women have an increased prevalence of the metabolic syndrome (138). The metabolic syndrome is strongly associated to diabetes, CVD (141)

and all-cause mortality (142) in the general female population, although it has been stressed that it is not more powerful in predicting CVD events than any other single risk factor (166). Hence, women with PCOS would be expected to have a greater risk of fatal and non-fatal CVD events during the postmenopausal period. However, due to the lack of prospective follow-up studies in postmenopausal PCOS women and the fact that the few existing studies show deficiencies, such as small sample sizes, wide age ranges, studies of women of young ages, cross-sectional designs, variations in PCOS criteria and different definitions and choices of outcome measures, evidence is still lacking.

Several biomarkers of CVD risk are increased in women with PCOS. Morphological markers has been found in PCOS women; for example, increased arterial calcification (in the carotid artery (increased intima media thickness) (151, 167, 168), in coronary arteries (169, 170) and in the aorta (170), and reduced vascular compliance (in the brachial artery, measured as flow-mediated dilation (171) or increased arterial stiffness (167, 168), in the internal carotid artery (172)). Reduced vascular compliance and increased arterial stiffness are signs of endothelial dysfunction, an early sign of atherosclerotic development, which has been found in the majority, but not in all studies of PCOS women (173). In addition, angiography has shown coronary artery disease to be associated with PCOS (174). However, all these studies are mainly dealing with young patients, in their thirties, with the exception of the studies by Christian et al., (patients up to 45 years of age)(169), by Talbott et al., 2000 (where 47 patients where aged >45 years, mean age 37.5±6.2 in all 125 PCOS)(151), and Talbott et al., 2004 (mean age 47.9±5.0) (170).

Increased levels of serum markers of CVD risk have been shown in women with PCOS. Thus, increased levels of, for example, plasminogen-activator inhibitor type 1 (PAI-I) (175-177), fibrinogen (177), (high-sensitivity) C-reactive protein (CRP) (178), and endothelin-I (168) have been found in PCOS women compared with controls. Numerous studies have shown an association in PCOS women between these abnormalities and insulin resistance and obesity (179). And, in general, PAI-1 is related to a prothrombotic state, whereas fibrinogen is a risk factor both for stroke and MI (180, 181), and elevated PAI-I and fibrinogen is associated with increased abdominal obesity (148). Furthermore, fibrinogen levels were found to be higher in obese PCOS than in lean PCOS women (134).

CVD morbidity and mortality

In 1987, Dahlgren and co-workers performed the first ever study of perimenopausal PCOS women (40-59 years) including 33 PCOS women and 132 age-matched controls. The women were not matched for BMI, but the groups were similar in this regard. The PCOS diagnosis in that study was entirely based on the typical histopathology of

specimens derived from wedge resections that all the included women had undergone in 1956-1965. The major findings were an increased prevalence of diabetes, hypertension, hirsutism and central obesity in the PCOS women. Moreover, the hormonal profile at this older age was typical of what had previously been found among younger PCOS women, with increased androgen and insulin levels and lower SHBG. Lower FSH was found in the PCOS women and, according to the menopausal levels of FSH, significantly more controls were postmenopausal (60% vs. 27%) (61).
A risk factor model concerning the risk of MI was derived from the independent risk factors found at that time for MI in a population study of 1462 women in Gothenburg (182), and this was applied on the same PCOS women and controls as in the study presented in 1992. Interestingly, this risk factor model predicted a 7.4 times increased risk of MI for the women with PCOS (183). Since the risk factor model included variables correlated to obesity it was concluded that obese/overweight PCOS women should be given advice by their physician "to be concerned about their weight, also for their future health."
Another small retrospective study of 28 previously wedge-resected women aged 45-59 years and their age-matched controls was published in year 2000; however, no differences regarding lipids or glucose levels were seen between PCOS and controls, although a higher prevalence of NIDDM and coronary artery disease was found in the PCOS women (184).

In 1998, a retrospective register-based long-term study on morbidity and mortality of PCOS women was published by Pierpoint et al. (122). Women with PCOS, diagnosed between 1970 and 1979 in the UK, were identified and 786 PCOS women were "followed" using records from general practitioners and by other records for an average of 30 years. The standard mortality rate, based on 59 deaths, was the same as the national rates for deaths due to circulatory disease and ischemic heart disease, whereas an increase in deaths due to diabetes was found, odds ratio (OR) 3.6, 95% confidence interval (CI): 1.5-8.4. However, the all-cause mortality was similar (122). In an investigation of the same cohort, Wild et al. (185), found a higher prevalence of diabetes, hypertension, hypertriglyceridemia and hypercholesterolemia (based on 61 women with PCOS/63 controls), but after adjustment for BMI, only the latter ramained. The standard mortality rate for all-cause mortality and cardiovascular mortality were similar to that for women in the general population. A history of coronary heart disease was not more common among the PCOS women. However, the crude OR (2.8, 95% CI: 1.1-7.1) for cerebrovascular disease was increased. The average age of the subjects in this study was 56.4 years (but with a wide range of 38-98 years) at follow-up and the BMI (27.1 kg/m^2) and WHR were higher in PCOS women than among controls.

The possibly increased CVD risk in PCOS has been extensively discussed in recent years and half of the documentation takes the form of review articles (186). A recent review on the subject (179) has summarized "long-term" studies examining the prevalence of CVD in women with PCOS and, in conclusion, the results of these studies are conflicting. Recently, the first meta-analysis on the subject was published (186) and described fatal/non-fatal coronary heart disease. A total of 1340 articles were identified, but only five met the inclusion criteria of the meta-analysis and the result indicated a relative BMI-adjusted CVD risk of 1.55. Three retrospective but only two prospective studies were included in the meta-analysis. The highest ranked study by Shaw et al. (178) was register based and 390 postmenopausal women who underwent a clinically indicated coronary angiogram were later examined regarding biochemical hyperandrogenism. The women were also asked about previous irregular periods. Of the 390 women, 104 were defined as having PCOS (age 62.5±10 years) and the CVD outcome was then followed for 6 years. The women with PCOS were reported to have angiographically verified coronary artery disease more often and lower cumulative event-free survival compared to those diagnosed as non-PCOS. In addition, a similar age at menopause was reported for both the PCOS and the non-PCOS women.

Taken together, prospective longitudinal studies of PCOS women in the postmenopausal period regarding sex hormones, CVD risk factors and events and mortality with a well-defined PCOS group are still lacking, as is knowledge about the ovarian pathophysiology and BMD/fractures. Future research on these issues is of utmost importance, in order to prevent future health problems in the large group of PCOS women.

Aims of the thesis

The overall aim of this thesis was to study some pathophysiological mechanisms in the ovary in women with PCOS of fertile age and to study systemic changes and their effect in postmenopausal PCOS women.

Specific aims were

Paper I — to investigate the difference in gene expression between women with PCOS and controls regarding selected genes considered to be of certain interest in the pathophysiology of PCOS.

Paper II — to study if women with PCOS differ from women in general regarding levels of reproductive hormones, anthropometry and presence of hirsutism/ climacteric symptoms also well after menopause and to compare changes in these variables in the same PCOS and control population during more than 20 years.

Paper III — to examine whether postmenopausal women with PCOS differ from controls concerning CVD risk factors, CVD events and mortality. Besides, the aim was to study changes in the CVD risk factors during a 21-year follow-up period.

Paper IV — to investigate whether postmenopausal women with PCOS differ from controls regarding body composition, BMD and fracture incidence during 21-year follow-up. In addition, the objective was to compare correlations between total BMD and sex hormones between women with PCOS and controls.

Subjects and methods

Ethics

All participants gave oral and written consent prior to inclusion in the studies of Paper I-IV. The studies were conducted in accordance with the Declaration of Helsinki and were approved by the Ethics Committee of the district of Copenhagen, Denmark (Paper I, GC) and by the Ethics Committee at the University of Gothenburg (Paper I, stroma, Paper II-IV).

Settings and study designs

The laboratory work in Paper I-IV, (except analyses of blood samples in the GC groups), was performed at the University of Gothenburg and Sahlgrenska University Hospital, Sweden. In Paper I, the stroma part from the ovaries of both PCOS women and controls originated from studies performed at the Reproductive Medicine Unit (PCOS stroma) and at the Gynecology Unit (control stroma) at Sahlgrenska University Hospital. In Paper I, the GC from both PCOS women and controls originated from women undergoing IVM treatment at the Fertility Clinic at Herlevs Hospital, Copenhagen, Denmark.
Paper I was composed of cross-sectional studies, comparing gene expression in PCOS women with gene expression in controls, in two different compartments of the ovary; the stroma and the GC.

The PCOS patients in Paper II-IV had undergone ovarian surgery (wedge resection) many years (in 1956-1965) before the studies. These surgeries had been performed in any of the three hospitals in the Gothenburg area at that time (Sahlgrenska, Mölndal and Östra). The controls of Paper II-IV were originally, in 1987, randomly allocated and age-matched to the PCOS women in a ratio of 4:1 and were taken from the WHO MONICA Gothenburg population study (182). The studies of Paper II-IV were controlled long-term follow-up studies, with the first examination performed in 1987. However, the baseline investigation of BMD was performed in 1992. The participating women, both the PCOS women and the controls, were re-examined in 2008, 21 years after the initial study (regarding BMD the follow-up was 16 years after the baseline investigation). The examinations were performed by the author and Dr Dahlgren, regarding the similar variables as in 1987. An overview of the study settings and patients can be seen in Table 2.

Table 2 Study setting/design and patients in Paper I-IV. The ages are presented as medians and ranges for Paper I-IV.

	Paper I	Paper II	Paper III	Paper IV
Setting	Stroma: Sahlgrenska GC: Herlev University Hospital → Sahlgrenska	Sahlgrenska		
Study design	Cross-sectional	Prospective follow-up study		
Study period	Stroma: 1999-2005 PCOS 2002-2004 Controls GC: 2004-2007 PCOS+Controls	21 years follow-up 1987 and 2008		
Number of women	Stroma: 7 PCOS 7 Controls GC: 5 PCOS 6 Controls	25 PCOS 68 Controls	25 PCOS 68 Controls Morbidity data PCOS: 32 Controls: 119 Mortality data PCOS: 6 Controls: 14	Bone measure PCOS 1992: SPA 20 Bone measure 2008: DXA, PCOS: 20 DXA, Controls: 66 Fracture data 2008: P: 25 C: 68
Age 2008 Median (range)	Stroma: PCOS 26.0 years (23.0-34.0) Controls 36.0 years (31.0-37.0) } p<0.005 GC: PCOS 28.0 years (22.0-34.0) Controls 32.5 years (29.0-34.0)	PCOS 70.0 years (61.0-79.0)# Controls 69.0 years (61.0-80.0) } NS		PCOS ¤ 68.0 years (61.0-78.0) Controls 68.5 years (61.0-80.0) } NS
Criteria	PCOS: Rotterdam Control: Non-PCOS	1) Stein-Leventhal → Rotterdam 2) Controls, random population sample WHO MONICA		

GC=granulosa cells; # median age and ranges for the patients participating in clinical examinations and questionnaire in 2008; ¤ median age and ranges for the patients participating in DXA 2008.

Patients and controls

General criteria of the PCOS populations (Paper I-IV)

All PCOS women in the studies of the present thesis met the Rotterdam criteria (9) and all were Caucasian. Patients with any other disorder mimicking PCOS were excluded before entering the study in 1987 (Paper II-IV) and the study of Paper I.

The PCOS women in the GC group and the stroma group (Paper I) all had PCO ovaries on ultrasound according to established criteria (9, 187). The PCOS women of the long-term follow-up studies (Paper II-IV) were all recruited many years before the Rotterdam consensus (9) and, thus, the PCOS diagnosis was originally based on histopathology (1, 188), on wedge resection specimens or unilaterally removed ovaries (because of benign ovarian disease). In 1987, the original histological slides of the ovarian tissue were re-examined by an expert pathologist and a correct histological diagnosis (defined as a

thickening of the tunica albuginea and outer cortex, multiple cystic follicles with prominent theca tissue, often with a tendency towards luteinization) was confirmed in 38 (all wedge-resected) of the 49 cases (see Fig 7). A strong correlation between PCO-like histology and the ultrasound diagnosis of PCOS has later been established (11, 12).

In addition to the PCOS ultrasound/histology criteria, one of the following two features had to be present (Paper I), or were present in 1987 (Paper II-IV), for the patients to be included in the PCOS group: 1) oligomenorrhea (with ≥35 days between cycles) or amenorrhea (absence of menstrual bleedings > 90 days), and/or 2) clinical or biochemical signs of hyperandrogenism. Clinical hyperandrogenism was defined by hirsutism, which was based on the patient's subjective estimation of the presence of excessive coarse hair (face, trunk, or thighs) and the need to remove hair at least twice a week. Good agreement has been confirmed between objective and subjective grading of hirsutism (11, 14). Biochemical hyperandrogenism in the stroma group (Paper I) was defined as total testosterone >2.85 nmol/L, and/or androstenedione >13.97 (age 20-29 years) or >7.32 (age 30-39 years) nmol/L, and/or DHEAS >9.79 (age 19-29 years) or >6.94 (age 30-39 years) μmol/L (calculated for all three androgens on the basis of their 95th percentile of the respective androgen levels). In the GC group, included in Denmark, the study protocol was unfortunately not fully followed and analysis of biochemical androgen variables was only performed in one of five PCOS women included. In Paper II-IV, biochemical hyperandrogenism was defined as total testosterone >3.16 nmol/L, and/or androstenedione >6.50 nmol/L, and/or DHEAS >7.20 μmol/L (calculated for all three androgens on the basis of their 95th percentile of androgen levels in 1987 of the control group).

General criteria of the control populations (Paper I-IV)

All the controls were Caucasian women with regular menstrual cycles. In addition, the controls in the stroma and GC groups (Paper I) were healthy and had normal ovaries on ultrasound (normal follicular distribution). None of the controls of the stroma and GC group in Paper I and had clinical signs of hyperandrogenism. The controls of Paper II-IV had no previous history of the combination of hirsutism and oligo-amenorrhea.

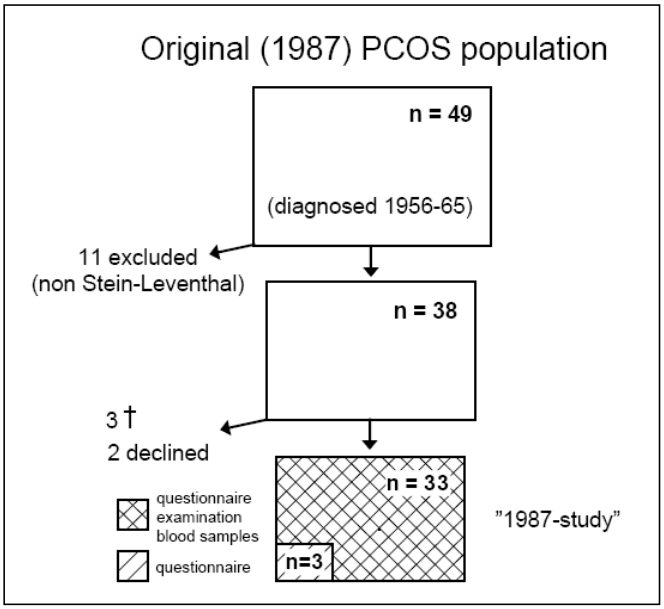


Fig. 7 Flow chart of the PCOS population of 1987. Forty-nine women were found to have been diagnosed (1956-65) with PCOS in the Gothenburg area. After histological re-examination 38 of these were found to have a correct diagnosis. Of these, three were already deceased and two declined to participate. Thus, 30 women with PCOS participated fully in 1987 and additionally 3 women participated only in the structured interview (questionnaire). This study population was re-examined in 2008 and the results are presented in Paper II-IV.

Stroma groups, PCOS and controls (Paper I)

PCOS

In Paper I, the PCOS women of the stroma group (Fig. 8) were included in a randomized study of infertility treatment of CC-resistant PCOS women, comparing ovarian drilling and low-dose gonadotropin therapy. The PCOS patients were referred to the Reproductive Medicine unit from the local hospitals in the Western Region in Sweden (VGR) for infertility > 1 year and were then asked to participate in the study if they had PCOS as a cause of infertility. Moreover, the patients had to be CC-resistant (i.e. no ovulations up to a dose of 150 mg CC for 5 days for 3 cycles or, if ovulating, no conception during at least 3 months), have a BMI ≤ 30 kg/m² and an age ≤ 40 years to be included in the study. The patients were examined by two doctors exclusively (Penilla Dahm-Kähler (P.D-K) or J.S). The patients could reach the two doctors at any time for questions. The inclusion of

patients started in 1999 and the study was closed at the end of year 2005, as the slow inclusion rate only had resulted in 21 patients being included during the study period of more than five years.

As the main purpose of this original study was for the patients to conceive naturally, they all underwent diagnostic laparoscopy to exclude tubal blockage, as the last part of their infertility investigation (Fig. 8). If no other causes of infertility were found during surgery the patients were randomized (sealed envelope method) during surgery to subsequent ovarian drilling or low-dose gonadotropin therapy (Fig.8). The sealed envelopes were prepared before the start of the study and 50% of the envelopes contained a paper saying "ovarian drilling" and 50% contained a similar paper with the text "gonadotropin therapy." The randomization was carried out by an independent laboratory technician, who was called if the patient was eligible for randomization. Nine patients were allocated to ovarian drilling, and as a part of the study protocol, they had also given their consent to providing a tissue sample from one of the ovaries if they were randomized to the group with ovarian drilling. There were some difficulties to obtain adequate tissue samples (see below) and the amount and quality of the tissue was only judged to be acceptable from seven patients out of nine patients. Seven of the nine patients who underwent ovarian drilling resumed ovulation (unpublished data). Two patients remained anovulatory, despite ovarian drilling, and these patients did not differ from those who resumed ovulation regarding anthropometry, age or sex hormone levels (including FSH, LH, androgens or SHBG).
Considering the exceptionally slow inclusion rate of patients in the study, the original study should, in the retrospect, have been designed as a multicenter study to have a chance of including enough patients to reach a statistical power of 80%. During the end of the inclusion period, a multicenter study on the subject were published, showing that ovarian drilling followed by CC treatment or gonadotrophin therapy, if anovulation persisted, was as effective as gonadotropin therapy (189). Although the sample size of seven patients in the PCOS stroma groups of Paper I is low, several human studies on gene expression profiles in gynecology associated areas have sample sizes of only five subjects (190, 191) (or even less) (192) or as few as two controls (193). Thus, the number of patients included in the stroma groups in Paper I should be regarded as good.

Controls
In Paper I, the controls providing the stroma originated from a study at the Sahlgrenska Academy, University of Gothenburg, investigating intra-ovarian ovulatory factors (194). This study was initiated in 2002 and the last patient underwent surgery in 2004. As a part of that study, tissue samples were taken from the ovary (see Materials and Methods,

Surgical techniques). These patients were initially referred to the Gynecology Unit, Sahlgrenska University Hospital, for laparoscopic sterilization. The patients were examined by three doctors exclusively (P.D-K, M.B and Anna-Karin Lind) and the patients included had to meet the general inclusion criteria for controls (see above) and the following other inclusion criteria: BMI <30 kg/m², age ≤40 years and no intake of any hormonal contraceptive ≤3 months prior to surgery. At the time of surgery the patients included in this study were in a preovulatory phase, defined as the presence of a dominant follicle with a size of 14 to 17.5 mm and before the spontaneous LH surge. To rule out premature luteinisation, estradiol and progesterone levels were also analyzed, to exclude patients with a low preovulatory estradiol or elevated progesterone level (194, 195). In total, 7 patients were included as controls in the study.

It should be pointed out that the menstrual cycle day of the stroma samples was well defined; however, it can of course be discussed which phase of the menstrual cycle would correspond best to the anovulatory ovary of the CC-resistant PCOS patients. It could be argued that a follicular phase with a dominant follicle of <9 mm may be more optimal.

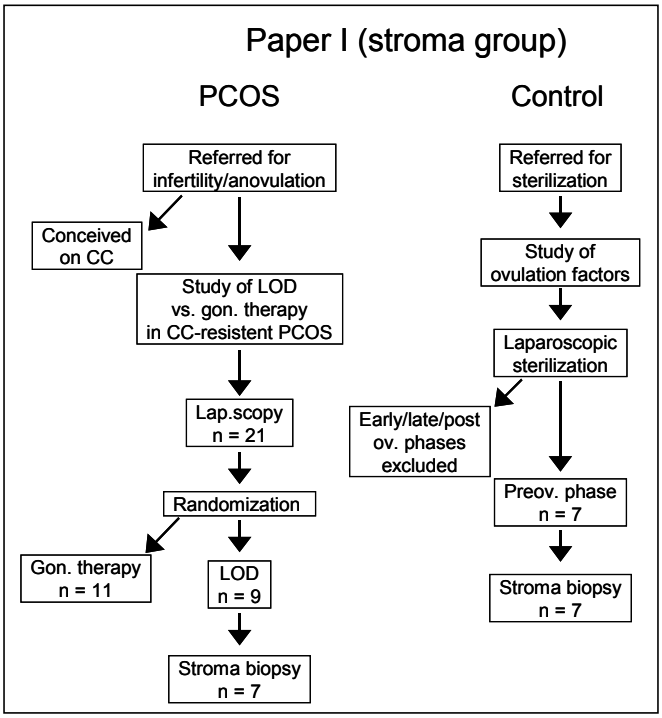


Fig. 8 Overview of the women with PCOS and the controls of the stroma study of Paper I. CC=clomiphene citrate, gon. therapy=gonadotropin therapy, lap.scopy=laparoscopy including test of tubal patency, LOD=laparoscopic ovarian drilling.

Granulosa cell groups, PCOS and controls (Paper I)

In Paper I, the PCOS women and controls providing the granulosa cells were participating in the IVM program at the Fertility Clinic, Herlevs University Hospital, Copenhagen, Denmark, to which they were referred for IVM due to male factor infertility, tubal factor or PCOS. All the PCOS women and the controls had to meet the general inclusion criteria of the respective groups (see above) and, in addition, both groups had to meet the following inclusion criteria: infertility ≥1 year, BMI 18-30 kg/m², age ≤40 years, no ovarian endometriosis, basal FSH <15 mIU/L and not >3 failures to conceive with IVF/ intracytoplasmatic sperm injection. All the PCOS women and controls were asked during the study period to participate if their treatment plan involved IVM. The inclusion of patients started in 2004 and the recruitment to the study was closed in 2007. Some initial samples were used for methodological studies of storage and transport of the samples. An overview of the used/analyzed samples of the PCOS and controls of the GC groups in Paper I is given in Fig. 9.

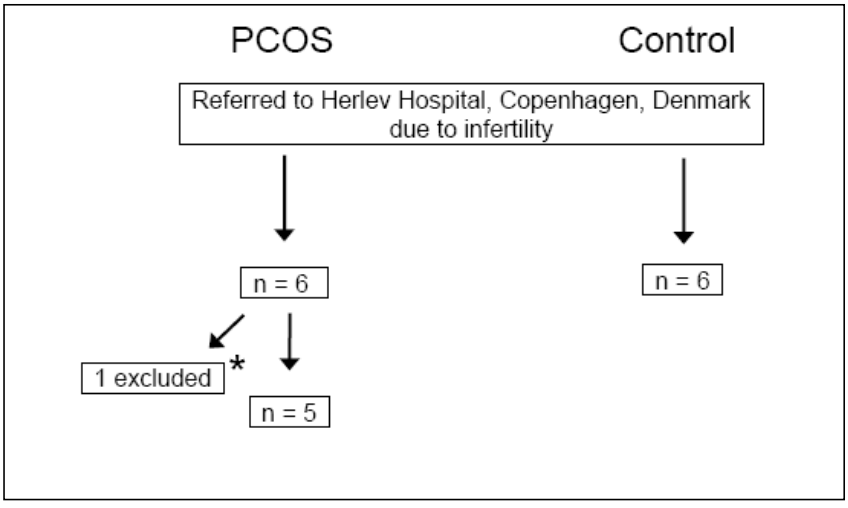


Fig. 9 Overview of the origin of analyzed granulosa cell (GC) samples from women with PCOS and controls of the GC group in Paper I. In the second set of samples sent from Denmark, GCs were received from 6 women with PCOS and 6 controls. *One PCOS woman was excluded as we could not confirm the PCOS diagnosis due to lack of hormonal data. Thus, data presented in Paper I regarding GCs are based on 5 PCOS women and 6 controls.

To further optimize the sub study regarding the gene expression in GCs, the PCOS group could have been better defined upon admission for IVM. Even though strict instructions were given regarding inclusion criteria and hormonal analyses, these criteria were not strictly followed. This fact emphasizes the problem of obtaining well characterized patient material, when the principle investigator is not directly involved in the contacts with patients.

Although all the PCOS women who were included met the Rotterdam criteria, the lack of data on possible biochemical hyperandrogenism is a limitation of the study. Other limitations are the mix of controls being primed with FSH and controls without priming. Another fact that has to be taken into account is the quite broad range regarding the cycle day of aspiration, varying from cycle day 8 to 18, compared with cycle day 7 to 9 in the PCOS group, although the median cycle days were similar. All these aspects may contribute to a difference in gene expression that we were unable to adjust for. Also, the mRNA quality of two of the samples was somewhat lower than the optimal level. That may be due to problems with the immediate handling of the sample, degradation during transport or changes during freezing. In an attempt to improve the conditions and thereby the quality of the RNA we repeated the on-site instructions to improve RNAse-free conditions at cell harvesting. We also tried to improve the quality by storing of the samples in RNA-later instead of in the frozen state during transportation; however the concentration of RNA in that set of samples was very low, (although the RNA quality was high), why those samples could not be used. To achieve better defined patients we asked for more patients/samples at the same time as the cells were put in RNA-later. In addition, the very first set of samples was not frozen upon delivery to Gothenburg and could not be used.

Taken together, all these factors made us decide to use the second set of samples (all samples had been frozen). Due to the uniqueness of the material we decided to finish the study anyway, with a restricted number (five) of cases. However, the number of patients included in the GC groups in Paper I should be regarded as acceptable although a larger sample size would have been advantageous (as stated regarding the sample size in the stroma groups). The study results may, however, open up for larger studies into this issue.

PCOS (Paper II-IV)

In 1987, 33 of the 38 women with a histopathologically verified PCOS diagnosis were included in the study by Dahlgren et al. An overview of the PCOS population in 1987 is given in Fig. 7.

Before the start of the analysis of the data from 2008, the original data of all the PCOS women from 1987 were reviewed in order to ensure that the patients were included

according to the Rotterdam criteria. Unfortunately, one patient had to be excluded. Also, the medical history from the structured interviews/questionnaires of 1987 and 2008 was reviewed, as were the results of the biochemical analyses in order to exclude disorders that could mimic PCOS.
In 2008, a few women had slightly elevated levels of prolactin and/or TSH, but were still included, as they were judged to have no clinical symptoms of hyperprolactinemia, and the elevations were so subtle that it was not considered to be true hyperprolactinemia, but probably caused by stress (maximum prolactin 883 mU/L). Regarding the elevated TSH levels, they were only slightly elevated in 2008 (maximum TSH 4.5 mU/L) and those patients were without any symptoms of hypothyroidism. Some of the patients with slightly elevated TSH levels were already treated with levothyroxine, the dose of levothyroxine was then corrected and levels were normalized.

In 2008, we re-examined the PCOS women and their controls regarding the following variables, as this was the first long-term follow-up of women with PCOS:

- Hormones and anthropometry (Paper II)
- CVD risk factors, CVD events, morbidity and mortality (Paper III)
- Body composition, BMD and incidence of fractures (Paper IV)

The number of patients, the studied subgroups and the phenotypes included in each subgroup are presented in Table 3.

Taken the long follow-up times and the high age of the women (median age 70 years, range 61-79) into consideration, the participation rate among the PCOS women (average participation rate ~78%) and the controls (average participation rate ~64%) should be regarded as highly acceptable.

Overviews of the studied PCOS women participating in the studies of Paper II-IV can be seen in Fig. 10 (Paper II), Fig. 11 (Paper III) and Fig. 12 (Paper IV).

Table 3 The number of patients, the studied subgroups and the phenotypes of the PCOS patients included in Paper II-IV. Data are given for the populations participating in 2008, except for in Paper IV, where data are also given for SPA (single photon absorptiometry) performed in 1992.

Paper n (%)	PCO+OA+HA n (%)	PCO+OA n (%)	PCO+HA n (%)	OA+HA n (%)
Paper I **Stroma n=7 (100)** **GC n=5 (100)**	4 (57) [c]	7 (100) 5 (100)	4 (57) [c]	4 (57) [c]
Paper II **Hormones/anthropometry n=25 (100)**	15[d] (60)	25 (100)	15[d] (60)	15[d] (60)
Paper III **Biochemistry n=25 (100)** **Morbidity n=32 (100)** **Mortality n=3 (100)**	15[d] (60) 18[a] (56) 2[b] (67)	25 (100) 30 (94) 2 (67)	15[d] (60) 18[a] (56) 2[b] (67)	15 [d](60) 18[a] (56) 2[b] (67)
Paper IV **SPA n=20 (100)** **DXA n=20 (100)** **Fractures n=25 (100)**	10 (50) 13[d] (65) 15[d] (60)	20 (100) 20 (100) 25 (100)	10 (50) 13[d] (65) 15[d] (60)	10 (50) 13[d] (65) 15[d] (60)

HA=hyperandrogenism clinical and/or biochemical; OA=oligo/amenorrhea; PCO=polycystic ovaries
[a] No information on 2 patients
[b] No information on 1 patient
[c] No data on biochemical hyperandrogenism in 4 GC patients; 1 woman was hirsute, 3 were not and no information was available regarding hirsutism for 1 woman
[d] No information on biochemical hyperandrogenism for 1 woman, but she had no hirsutism

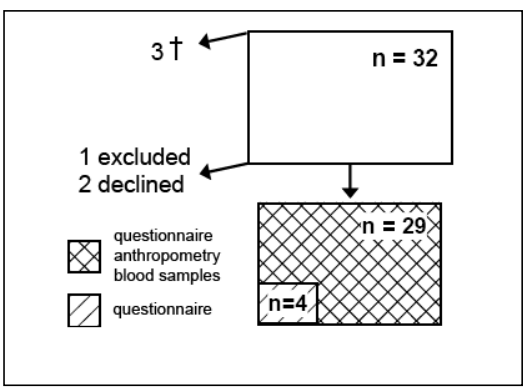


Fig. 10 Overview of the PCOS women of Paper II. In 2008, it was possible to contact thirty-two of the PCOS women (in total 6, of the originally 38 women with PCOS were deceased). Twenty–nine of these women participated in a structured interview based on a questionnaire and 25 of them were also investigated by blood samples and examination.

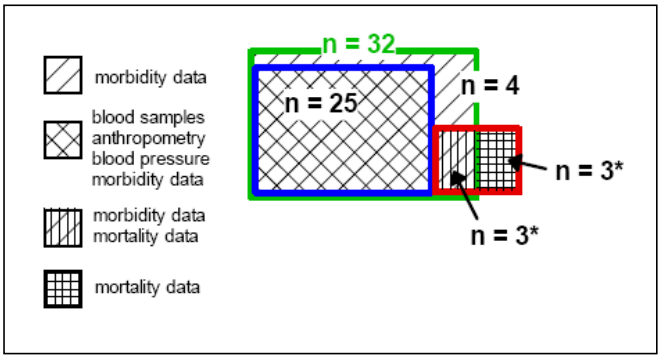


Fig. 11 Overview of the PCOS women of Paper III. In 2008, 25 women participated fully in blood sampling, examinations (anthropometry and blood pressure) and structured interviews (questionnaire). Four more participated only in the structured interview (questionnaire). Six women were deceased and, of these, morbidity and mortality data were obtained for three women and mortality data only for the other three.

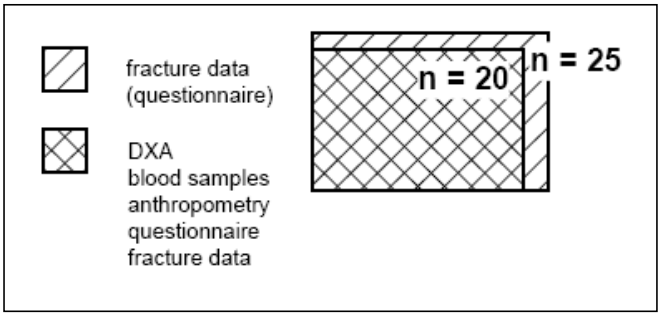


Fig. 12 Overview of the PCOS women of Paper IV. In 2008, 25 women with PCOS participated in the structured interview, blood sampling and anthropometric measures. Twenty of these women also participated in the DXA examination. In paper IV, the presented fracture data presented were based on 25 PCOS women and the DXA data on 20 of these women. To give background data on the patients participating in the DXA examinations, data on anthropometry and sex hormones for these 20 patients were also presented.

Controls, the WHO MONICA population study (Paper II-IV)

The controls of Paper II-IV were randomly allocated and age-matched to the PCOS women in 1987, originally in a ratio of 4:1, from the population World Health Organisation (WHO) population study MONItoring of trends and determinants for CArdiovascular disease (MONICA) Project, Gothenburg, Sweden (182). The MONICA Project is a screening study for CVD risk factors and comprises 38 centers around the

world. In the MONICA screening in Gothenburg, Sweden, performed in 1985, 1000 women and 1000 men aged 25-64 years were selected at random from the population of Gothenburg and were invited to participate (182).
In 2008, the original data of the controls participating in the study in 1987 were reviewed. The patients were excluded if they had biochemical hyperandrogenism or hirsutism in the data of 1987 in combination with a statement in the questionnaire in 1987 that they had had irregular menstruations or amenorrhea. After this review one control woman was excluded from the data of 2008 and 1987, as she had reported irregular periods and hirsutism.
In addition to being age-matched, the controls were also similar to the PCOS women regarding BMI, both in 1987 and in 2008. The similarity in BMI is vital when studying hormones, coagulation factors, CVD risk factors and BMD, as all of these factors may be dependent in part on the BMI (Introduction). Subgroups of controls and the number of women in each subgroup are shown in Table 2.

Non-attendants (Paper II-IV)

In Paper II and III, there were only two PCOS women who did not participate and declined to participate in both 1987 and in 2008. In the non-participating controls of Paper II and III, no differences were shown regarding anthropometric measures or the prevalence of any studied morbidity compared to the participating controls in 2008.

In Paper IV, data on the fracture incidence in non-attendants and deceased subjects were not available. Of the 25 PCOS women participating fully in Paper IV, five did not want to take part in the DXA examinations. These five women were older and heavier than the participating PCOS women. Otherwise, no differences were seen regarding the prevalence of diseases or in incidence of fractures. Only two of the controls who participated in the structured interview, blood sampling and anthropometric measures in 2008 declined to take part in the DXA examinations.

Material and methods (Paper I)

Surgical techniques (Stroma groups), PCOS and controls

PCOS

In paper I, the PCOS women who were allocated to ovarian drilling had also consented to provide a small cylindrical ovarian biopsy. This was taken before the ovarian drilling and through the entire ovary using a customised stainless steel cylinder (inner diameter 3 mm, outer diameter 4 mm, length 40 cm; Meditech, Institute of Neuroscience and Physiology, University of Gothenburg) with sharp edges (Fig 13). The ovarian biopsy was extracted from the steel cylinder by a piston and examined for macroscopic tissue quality. Each ovary was punctured by unipolar diathermy (40W) at ten to fifteen sites according to the original report and personal instructions by Gjonnaess (83). The protocol was to include only biopsies that were obtained as a single cylinder of tissue and to separate and discard the outer 1 cm at each edge, which would contain any follicular tissue and/or surface stroma. The central part (mostly stroma tissue) was frozen approximately 10 min after the biopsy was performed and stored at –80ºC until total RNA was extracted.

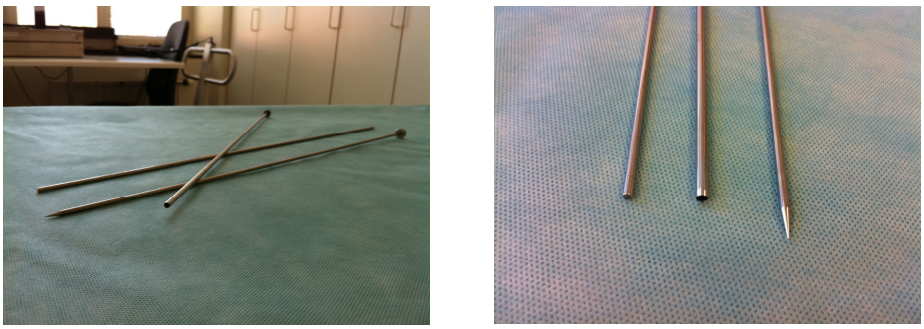


Fig. 13 Photo of the customized drill and the piston.

Controls

The control women of Paper I who underwent laparoscopic tubal sterilization had also consented to provide a small ovarian biopsy. This was taken before the sterilization by excising the whole dominant follicle using scissors that cut through the stroma surrounding the preovulatory follicle and by removing the intact follicle inside a laparoscopic sac via a small suprapubic incision. In this follicular sample there would always be a rim of stroma tissue on the basal side of the follicle (Fig. 14). Diathermy was not used, as this would most likely cause thermal damage to the tissue, with predicted

degradation also of the mRNA and proteins. The removed tissue was then placed on ice and brought to the laboratory for dissection. After follicle retrieval, sterilization was performed. The stroma tissue of around 3-4 mm at the base of the follicle was dissected from the true follicular wall and used in the present study. Based on the normal position of a human preovulatory follicle (195-197) within the human ovary, this tissue would correspond to the central stroma (Fig 14). The tissue samples were snap frozen and kept at -80ºC until RNA preparation.

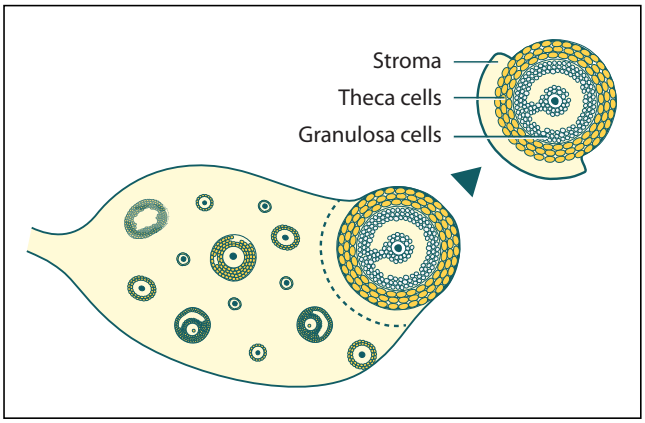


Fig. 14 Schematic drawing of the tissue separation of the human follicle after surgery with the purpose of obtaining stroma from the controls in Paper I.

In vitro maturation (GC groups), PCOS and controls

The PCOS women who underwent *in vitro* maturation were treated for three days with 150 IU recombinant FSH (Gonal-F®, Serono, Geneva, Switzerland). This treatment was initiated on day 2-3, after spontaneous or induced menstruation. Oocyte aspiration was performed on day 8-9 after deprivation of FSH for 2-3 days. Two controls were treated with FSH and had the same timing of aspiration, while the remaining four controls underwent non-stimulated cycles. In these cycles, oocyte aspiration was performed on the day after detection of a dominant follicle of 10 mm, with the follicular diameter being calculated as the mean of the longest follicular axis and the axis perpendicular to that.

All cycles were monitored by ultrasound on day 2-3, day 6-7 and thereafter with an interval of 1 or 2 days until oocyte aspiration. The cycle was cancelled if an ovarian cyst > 10 mm was observed on day 3 of the cycle. Oocyte pick-up was performed transvaginally with a 17G single lumen needle (K-OPSC-1225; Cook, Brisbane, Australia) connected to a syringe to induce aspiration vacuum. To remove erythrocytes and cellular debris in order to obtain the oocytes more easily, the follicular fluids were filtered (Falcon 1060, 70 µm mesh size). The follicular fluids were then centrifuged (3000g, 5 minutes).

The pelleted GCs were immediately frozen in liquid nitrogen and stored at -80ºC until transportation on dry ice and further analysis in Gothenburg.

RNA extraction and quality

In Paper I, all experiments were performed under RNase-free conditions. The first step was to isolate the mRNA from the tissue (Trizole, Invitrogen, Carlsbad, CA, USA, was used for this, according to the manufacturer's protocol). The RNA concentrations were then measured (Nanodrop, Thermo Fisher Scientific, Wilmington, DE, USA) and the quality of the extracted mRNA was then assessed (Agilent 2100 Bioanalyzer, Agilent technologies, Kista, Sweden). The quality of the extracted mRNA is expressed as the RNA integrity number (RIN). A good correlation between RIN values above 5.5 and the outcome of a real-time PCR experiment has been shown (198), and RIN values ≥5.5 are considered to be high-quality values (198). In the stroma, all the samples had RIN values showing high-quality RNA, as did the GC samples from the controls. However, two samples of GCs from PCOS women had lower RIN values (5 and 4). As it proved to be difficult to include patients in the studies we decided to keep the sample with a RIN of 4 in the study and if the CT value of that specific sample differed considerably in range from the other samples it would later be excluded. As this was not the case, this specific sample remained included.

Quantitative real time polymerase chain reaction (QPCR)

The QPCR was used in Paper I. The QPCR is highly sensitive and enables quantification of small variations in gene expression. The process is performed in two steps, where the first step is to synthesize complementary deoxyribonucleic acid (cDNA) from each mRNA molecule using the enzyme reverse transcriptase (reverse transcription); a necessary step as RNA removed from its environment is not stable enough for PCR and would thus degrade rapidly. In the second step, the gene of interest (the target gene) and the endogenous control (the housekeeping gene) are amplified by the PCR from the cDNA mixture, including primers and a probe complementary to the target sequence. The endogenous control is an included reference gene whose expression should be as stable as possible; *i.e.,* as unaffected as possible by the conditions of the investigation. The probe is labeled with a 5`end reporter dye and a 3`end quencher. The result is increased fluorescence as the probe dye is cleaved and separated from the quencher by the exonuclease activity of the DNA polymerase. The fluorescence is detected and increases as the amplification progresses and the instruments register the amount of cDNA during each amplification cycle. The cycle at which the instrument can discriminate between the amplification-generated fluorescence and the background noise is called the threshold cycle (C_T). The quantity of DNA theoretically doubles every cycle during the exponential

phase and relative amounts of DNA can be calculated *i.e.,* a sample with a C_T 3 cycles earlier than another has 2^3=8 times more templates. Thus, in general, amplification of a cDNA follows a curve with an initial flat phase, followed by an exponential phase and when the experiments reagents are used up, the DNA synthesis slows down and the curve evens out to a plateau.
After control for similar amplification efficacy of the target genes and the endogenous control, the relative expression was presented using the comparative $2^{-\Delta\Delta CT}$ method (199). In that method the amount of target gene is normalized to the endogenous reference gene and related to a control (calibrator). At first, the C_T values of both the samples and the controls are studied and normalized to the most appropriate endogenous control/s chosen.

The ΔC_T sample is given by: $\Delta C_T = C_T$ target - C_T endogenous control

Then ΔC_T is converted to relative quantities displaying the $\Delta\Delta C_T$; *i.e.*, the cycle threshold differences after normalizing to the endogenous control and to the control/calibrator.

The $\Delta\Delta C_T$ is given by: $\Delta\Delta C_T = \Delta C_T$ sample - ΔC_T calibrator

In this equation, the target and calibrator could be treated and untreated samples, respectively, or in the case of paper I, PCOS samples and normal samples.

In the last step $\Delta\Delta C_T$ is converted to fold changes "amount of target"=$2^{-\Delta\Delta CT}$.

In paper I, Taqman Low Density Array (TLDA, Applied Biosystems) was used. We had selected 57 genes (target genes) that were considered to be of specific interest to the pathophysiology of PCOS. A customized 64-well plate was chosen, to enable 64 simultaneous QPCR reactions. The target genes and endogenous controls were taken from inventoried Taqman expression assays (Applied Biosystems) and were factory-loaded onto each plate.
We chose seven genes as possible endogenous controls. The Normfinder algorithm, version 0.953 (http://www.mdl.dk/publicationsnormfinder.htm), was used to select the most appropriate stable endogenous controls. The analyses were run on a 7900-HT Fast Real Time PCR System (Applied Biosystems) according to the manufacturer's instructions, at the Genomics Core Facility at the University of Gothenburg. The data were analyzed using the SDS (Sequence Detection Systems) software (Applied Biosystems). Baseline and threshold levels for each gene were set automatically using the Auto C_T algorithm. All reactions were performed in triplicate and means of these values were used for the statistical analyses.

Interpretation of QPCR outcome

For bioinformatics; GeneCards®, version 3 (www.genecards.org), PubMed, and The Ovarian Kaleidoscope Database (http://ovary.stanford.edu) were used as sources of information.

Histology

A minimum piece of some of the biopsies (where good, larger biopsies were obtained) of the PCOS and control stroma were fixed in 4% phosphate-buffered formaldehyde, embedded in paraffin, sectioned and stained with hematoxylin and eosin. Light microscopy of the histological slides was performed to examine whether the tissue architecture of the stroma in the PCOS and control group was similar.

Material and Methods (Paper II-IV)

Terms and definitions

Age at menopause was defined as the time of the final menstrual period followed by amenorrhea of at least 12 months. The calculated age at menopause was calculated after exclusion of hysterectomized women <45 years of age (PCOS 16%, Controls 4%). Hypothyroidism was defined as TSH ≥4.5 mU/L and/or use of levothyroxine, and hyperprolactinemia as prolactin ≥400 mU/L. Anti-thyroid peroxidase (TPO) was considered to be positive if levels were ≥60 kU/L (according to the reference value of our laboratory).

Hypertension was defined as the presence of an International Classification of Diseases (ICD) diagnosis of hypertension and/or by the use of an antihypertensive agent. Diabetes was defined as a diagnosis of type 1 or type 2 diabetes and/or medical or dietary treatment for diabetes. MI and stroke were defined as being hospitalized due to the diagnosis according to the ICD, with only the first respective event being counted. Smoking was defined as current or never smoker. A sedentary lifestyle was defined as almost complete inactivity; for instance, watching TV. Moderate exercise was defined as walking, cycling or light gardening at least 4h/week. Regular exercise was defined as regular more strenuous activity; i.e., running or heavy gardening >2-3 h/ week. Strenous activity was defined as regular athletic training and/or participation in competitive sports several times/week.

Osteoporosis was defined according to the WHO criteria (101) with a T-score ≤-2.5 SD of young adults at either the lumbar spine or femoral neck. Fractures were defined as X-ray-verified fractures, as asked for in the questionnaire. Osteoporotic fractures were defined as wrist, hip and vertebral fractures occurring at an age >40 years with high-energy traumatic fractures excluded and only the first fracture included as a data point.

Anthropometric measures

Body height and weight were measured to the nearest 0.1 cm and kg in the fasting state, with the patients in underwear. BMI was calculated as body weight divided by height squared (kg/m²). Waist and hip circumferences were measured with a soft tape in the standing position to the nearest cm at the umbilicus and over the widest part over the gluteal region, and the waist/hip ratio was calculated.

Blood pressure

Blood pressure was measured on the right arm in the sitting position after 15 min of rest to the nearest 2 mmHg. A cuff size corresponding to the circumference of the right arm was chosen. In 1987, a mercury sphygmomanometer was used and disappearance of Korotkoff sounds (phase V) was used to determine diastolic pressure. In 2008, an automatic sphygmomanometer (Omron M7, Intelli Sense™, Omron Healthcare, Kyoto, Japan) was used.

The presented blood pressures should be interpreted with caution as the blood pressure should be measured at three separate occasions after 15 min of rest according, to the established recommendations.

Questionnaire

All the women were interviewed about their medical history, family history, social and lifestyle factors as well as present medication, according to a structured questionnaire. The questionnaires were identical in 1987 and 2008, except the parts regarding stress and fractures being added in 2008. The questionnaire, including degree of stress and physical activity, have been validated and used before in other WHO MONICA population studies (182, 200, 201).

Registry Data

Data from the National Board of Health and Welfare Registry provided morbidity and mortality data on the diseased women. Data on morbidity caused by MI, stroke, hypertension, diabetes and cancer were derived from The Hospital Discharge Registry, coded according to ICD, version 9 and 10.

Single photon absorptiometry (SPA)

In 1992, the general methods to assess BMD was by the use of SPA measured in the distal non-dominant forearm (Osteometer DT 100, A/S, Rodovre, Denmark), which was also used by us and by established osteoporosis researchers from our region (102). These methods were used in 1992 only on the PCOS women and, unfortunately, no

measurements were made in controls at that time. The BMD results from 1992 were not published at that time.

Dual energy X-ray absorptiometry (DXA)

In 2008, lean body mass, adipose tissue and BMD were assessed by DXA (Lunar Prodigy DXA, GE Lunar Corp., Madison, WI, USA), Fig. 15.

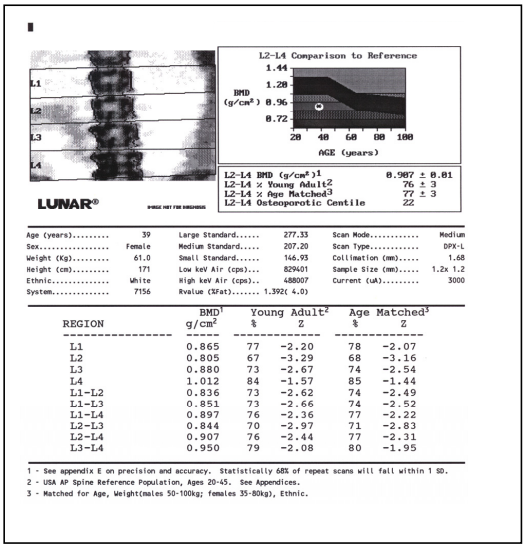


Fig. 15 DXA measurement using the Lunar Prodigy DXA and DXA protocol.

Biochemical analyses in 2008 *(Paper I-IV)*

The blood samples in the stroma groups (Paper I) were taken immediately before surgery. The blood samples of the stroma group were analyzed at the laboratory at the Department of Obstetrics and Gynecology at Sahlgrenska University Hospital, Gothenburg, Sweden. The blood samples of the GC group in Paper I were analyzed at the Laboratory of Clinical Biochemistry at Herlevs University Hospital, Denmark.

In Paper II-IV, fasting venous blood samples were drawn from an anticubital vein between 0700 and 1000h. The samples were stored at -70ºC until analyzed (within 6 months). All biochemical analyses, except for androstenedione and estrone, were performed by the Laboratory for Clinical Chemistry, Sahlgrenska University Hospital (accredited, SWEDAC ISO 15189). Analysis of androstenedione and estrone were

performed at the research laboratory of the Department of Obstetrics and Gynecology at the same hospital. The assays used in 1987 and the corresponding data have been presented elsewhere (61, 175, 183). Biochemical assays identical to those used in 1987 were unfortunately not available in 2008.

All coefficients of variation (CV) of the different assays are stated below, and unless otherwise specified, the CVs are the total CVs including both the intra-assay and inter-assay variation.

Pituitary hormone assays

In paper I (stroma group), serum LH, FSH and prolactin (only in paper I), were measured using chemiluminescent microparticle immunoassay (CMIA) (Architect Reagent Kits; Abbott Laboratories, Diagnostic Division, Abbott Park, IL, USA). In paper I (stroma group), the LH assay had a detection limit of 0.07 mU/mL, with CVs of 4.1 at 5.2 mU/L and 3.3% at 39.2 mU/mL. The FSH assay had detection limit of 0.05 mU/mL with CV< 4.6% at 5.6 mU/mL, <3.8% at 25.1mU/mL and <4.11% at 74.7 mU/mL. In paper I (GC group), LH and FSH were measured using CMIA (ADVIA Centaur kits, Siemens, Terryton, NY, USA). The LH assay had a detection limit of 0.07 mU/mL with CVs of 2.7% at 4.2 mU/L and 3.8% at 21.7 mU/mL. The FSH assay had a detection limit 0.3 mU/mL with CVs 3.9% at 6.9 mU/L, 2.8% at 12 mU/L and 2.9% at 144mU/L.

In paper II and IV, FSH, LH and prolactin were measured using CMIA kits from the same manufacturer as in paper I (stroma group) but with different detection limits and CVs. This LH assay had a detection limit of 0.07 IU/L and CVs of 11% at 3 IU/L, 8% at 20 IU/L and 7% at 60 IU/L. The FSH assay had a detection limit of 0.05 IU/L and CVs of 9% at 8 IU/L, 6% at 16 IU/L and 4% at 40 IU/L. The detection limit of the prolactin assay was 0.1 mIU/L with CVs of 10% at 175 mIU/L, 6% at 350 mIU/L and 7% at 900 mIU/L. TSH was analyzed using an electrochemiluminescence immunoassay (ECLIA) (Roche Diagnostics, GmbH, Mannheim, Germany). The detection limit was 0.0051 mIU/L, with CVs of 7% at 0.4 mIU/L, 6% at 4 mIU/L and 5% at 20 mIU/L

Steroid hormone assays

In paper I (stroma group), total testosterone, androstenedione and DHEAS were measured using radioimmunoassay assay (RIA) kits (muChem™Double Antibody Testosterone 125I RIA Kit, ICN Biomedicals, Inc, Costa Mesa, CA, USA; Coat-A-Count® Direct Androstendione and DHEAS kits; Diagnostic Products Corporation, Los Angeles, CA, USA). The total testosterone assay had a detection limit of 0.3 nmol/L with a CV of 10% at 2, 15 and 25 mmol/L. The androstenedione assay had a detection limit of 0.5 nmol/L

with CVs of 15% at 3.2 and 10% at 11.2 nmol/L. The DHEAS assay had a detection limit of 0.14 µmol/L with a CV of 12% at 3 and 10 µmol/L. In paper I (stroma and GC group) serum estradiol and progesterone were measured using CMIA. In the stroma: (Architect Reagent Kits; Abbott Laboratories, Diagnostic Division, Abbott Park, IL, USA). The estradiol assay had a detection limit of 36.7 pmol/L with CVs of 6.6% at 45 pg/mL and 2.3% at 192 pg/mL. The progesterone assay had a detection limit of 0.3 nmol/L and CVs of 19, 11 and 13% at 2.7, 27 and 70 nmol/L, respectively. In the GC group of paper I, (kits from ADVIA, Centaur kits, Siemens, Terryton, NY, USA) was used. The estradiol assay had a detection limit of 25.7 pmol/L with CVs of 12, 7.1 and 11.4% at 206.6, 392.3 and 2076.1 pmol/L, respectively. The progesterone assay had a detection limit of 0.67 nmol/L and CVs of 12.7, 5.4 and 3.7% at 3.8, 22.9 and 66.5 nmol/L, respectively.

In paper II and IV, RIA kits were used for analysis of DHEAS (Siemens Diagnostics Products; Los Angeles, CA, USA, estradiol (Clinical Assays; Sauggia, Italy) and total testosterone (Beckman-Coulter, Fullerton**,** CA, USA). The DHEAS assay had a detection limit of 0.14 µmol/L, with CVs of 12% at 3 µmol/L and 10 µmol/L. The estradiol assay had a detection limit of 0.04 nmol/L and CVs of 10% at 0.4 nmol/L and 16% at 0.04 nmol/L. The total testosterone assay had a detection limit of 1.0 nmol/L and CVs of 10% at 2, 15 and 25 nmol/L. In paper II and IV, estrone and androstenedione were measured using ELISA kits (DRG International Inc., Mountainside, NJ, USA). The estrone assay had a detection limit of 23.3 pmol/L, intra-assay CVs of 9% at 55.5 pmol/L, 8.5% at 406.9 pmol/L, 4.5% at 980.2 pmol/L and inter-assay CVs of 13% at 55.5 pmol/L, 7.5% at 418.0 pmol/L and 7% at 1039.3 pmol/L. The androstenedione assay had detection limit of 0.07 nmol/L, and intra-assay CVs of 9% at 1.05 nmol/L, 5.6% at 9.08 nmol/L and 4.7% at 16.4 nmol/L. The inter-assay CVs of the androstenedione assay were 9.6% at 0.70 nmol/L, 12.1% at 8.03 and 8.8% at 15.6 nmol/L.

SHBG assay

In paper I, serum levels of SHBG were performed using immunoradiometric analysis (Orion Diagnostica, Espoo, Finland). The SHBG assay had a detection limit of 0.5 nmol/L and CVs of 7% at 40 and 5% at 60 nmol/L.

In paper II and IV, SHBG levels were determined using CMIA kits (Biokit; Abbot Laboratories, Diagnostic Division, Barcelona, Spain). This SHBG assay had a detection limit of 0.1 nmol/L with CVs of 7% at 20 and 40 nmol/L and 9% at 100 nmol/L.

Insulin assays

In paper I (stroma group), serum levels of insulin were measured using a RIA kit (Pharmacia Insulin RIA 100; Pharmacia Upjohn Diagnostics AB, Uppsala, Sweden). The insulin assay had a detection limit of 3.0 mU/L and a CV of 10% at 10 and 40 mU/L.

In paper III, serum levels of insulin were measured using CMIA (Insulin Elecsys, Roche Diagnostics, Mannheim, DE) with a detection limit 0.2 mU/L and CVs of 10% at 6, 20, 70 mU/L.

Glucose assay

In paper I and III, a hexokinase-based photometric method GLU (Roche, Diagnostics GmbH, Mannheim, Germany) with a detection limit of 0.11 mmol/L and CVs of 4% at 5 and 15 nmol/L was used to analyze plasma glucose.

Anti-thyroid peroxidase (TPO) assay

In paper II, immunofluorescence (TRACE technique; Brahms AG, Henningsdorf, Germany) was used for quantitative determination of auto-antibodies against TPO/microsomal antigen (anti-TPO), with a detection limit of 0.01 kU/L and CVs of 19% at 100, 7% at 600, 10% at 2000 kU/L.

Blood lipid assays

In paper III, enzymatic, photometric methods were used to analyze serum levels of total cholesterol, high-density lipoprotein (HDL) cholesterol, low-density lipoprotein (LDL) cholesterol and triglycerides. The detection limits for total cholesterol, HDL and LDL assays (Roche Diagnostics, GmbH, Mannheim, DE) were 0.08 mmol/L. The CV of the cholesterol assay was 3% at 4 and 6 mmol/L. The CV of the HDL assay was 5% at 1 and 2 mmol/L. The CV of the LDL assay was 4% at 2 and 5 mmol/L. The triglyceride assay had a detection limit of 0.05mmol/L with a CV of 4% at 1 and 2 mmol/L.

Immunoprecipitation, enhanced by polyethylene glycol (Thermo Fisher Scientific, Vantaa, Finland), was used to determine Apo A1 and Apo B with detection limits of 0.1 g/L and 0.05 g/L, respectively, and CVs <5%.

Coagulation assays

In paper III, ELISA was used to measure plasminogen activator inhibitor-1 (PAI-1) antigen (Trinity Biotech, Jamestown, NJ, USA) with a detection limit of 2 µg/L and CVs of 9% and 12% at 13 and 50 µg/L, respectively. Fibrinogen was determined using a commercial assay (Diagnostica, Stago Asnières, France) according to von Clauss (202) The detection limit was 0.6 g/L, with CVs of ≤7%.

Indices

Free androgen index (FAI)

In Papers II-IV, FAI was defined as total testosterone/SHBG x 100.

HOMA

In Paper III, HOMA was defined as fasting blood glucose x serum insulin/22.5 (124).

Statistics (Paper I-IV)

Statistical analyses were performed using Prediction Application Software (version 18.0 for Windows; SPSS, Chicago, IL, USA). A p-value <0.05 was considered significant. Means, SDs, medians and ranges were calculated with conventional methods.

In Paper I intergroup comparisons were made using the non-parametric Mann-Whitney U-test. The median fold change was given by the ΔΔCT of PCOS, divided by the ΔΔCT of controls for the over-expressed genes and by the ratio of ΔΔCT of controls to ΔΔCT of PCOS for the under-expressed genes.

General statistical tests of Papers II-IV:

In Paper II-IV, the following statistical tests were used in all studies, (some specific tests were used in each study, as specified in the next paragraph). Intergroup- and intragroup comparisons were performed using the non-parametric Mann-Whitney U-test and the Wilcoxon ranked pair test, respectively. Comparisons between groups regarding distributions were performed using the Chi-square test. When the lowest frequency in any of the cells was <10 (2 by 2 table), Fisher's Exact Probability Test was used instead. Comparisons of symptoms classified by a scale were analyzed using test for trend in contingency table.

In Papers II and III, the results are presented as means and SDs although non-parametric tests were used, due to the requirements of the journal regarding the number of allowed column tables and the layout. In papers I and IV, results are presented according to the convention when non-parametric tests based on ranks were performed with medians, ranges, or, more specifically, minimums and maximums, in order to describe the ranges in a better way.

Specific statistical tests of any of Papers II-IV

In Paper II and III, changes were defined as the value in 2008 minus the value in 1987.

In Paper II, test of correlation between age and FSH was done using Pitman's test and illustrated by the use of linear regression.

In Paper III, a retrospective power calculation was performed based on the observed differences in the presented study, for the purpose of achieving an 80% power, at a significant level of 0.05. The expected number of deaths was calculated based on the assumption that the death hazard function coincided with that of women in the general Swedish population. Current age and calendar time were taken into account and p-values and exact 95% CIs of hazard ratios were determined using Poisson distributions. The last calculation was based on time from wedge resection to the end of 2010.

In Paper IV, correlations were tested using the non-parametric Spearman's test. A comparison between groups regarding correlation coefficients was performed by applying Fisher's z transformation.

A summary of the statistics and types of statistical tests used in Paper I-IV is shown in Table 4.

Table 4 Summary of the statistics used in Paper I-IV.

	Paper I	Paper II	Paper III	Paper IV
Sign. level	← 0.05 two sided test →			
Analytical statistics	- Mann Whitney U-test - $\Delta\Delta C_T$ PCOS/ $\Delta\Delta C_T$ controls for over-expression - $\Delta\Delta C_T$ controls/ $\Delta\Delta C_T$ PCOS for under-expression	- Mann Whitney U-test - Wilcoxon ranked pair test - Chi-square test - Fisher Exact Probability - Test for trend in contingency table - Pitmans test	- Mann Whitney U-test - Wilcoxon ranked pair test - Chi-square test - Fisher Exact Probability - Test for trend in contingency table - Poisson distributions	- Mann Whitney U-test - Wilcoxon ranked pair test - Chi-square test - Fisher Exact Probability - Test for trend in contingency table - Spearman's rank correlation - Fisher's z-transformation

Results and comments

Differential expression of inflammation-related genes in the ovarian stroma and granulosa cells of PCOS women (Paper I).

Several pathophysiological aberrations exist in the ovaries of PCOS women, with the characteristic functional alterations being increased production of androgens with the accompanying morphological changes, especially in the androgen-producing theca layers of the antral follicles and the interstitial and capsular stroma.

Naturally, PCOS and its pathophysiological consequences have mostly been studied from a clinical perspective, with studies looking into changes in hormonal levels, body composition, anthropometry and symptoms, such as hirsutism, obesity, etc (6, 11, 34). Tissue samples from PCOS women are much more difficult to obtain than blood samples and external values, but one type of internal tissue that is obtainable with a low degree of invasiveness in the harvesting procedure is peripheral fat tissue. Thus, during the last few years, a few studies regarding the PCOS pathophysiology have been performed on adipocytes from peripheral fat tissue (203, 204).

Concerning ovarian samples from PCOS women and related studies on ovarian gene expression, a very restricted number of publications have been presented during the last decade, typically with small (n= 3-8) sample sizes (95, 190, 192, 193). This is due to the inaccessibility of the intra-abdominally situated ovary and the need for major invasive procedures to obtain the material. Previous functional experiments on ovarian tissue/cells have mostly used GCs and theca cells from occasional PCOS follicles obtained at hysterectomy surgery (43) or at follicular aspiration (50), and comparable tissue samples from several PCOS and non-PCOS women, which would allow for proper comparisons, have been scarce. These studies have been driven by the specific hypothesis that the ovarian cells of PCOS women would react differently from normal cells to certain hormones or other perturbants. Gene arrays have made it possible to examine a small cell/tissue sample with respect to the global gene expression. In the field of ovarian pathophysiology in PCOS, there are to my knowledge, five studies on global gene expression, using either cultured PCOS theca cells (205), whole ovarian tissue (95, 192), oocytes (206) or cumulus cells (207). These studies have given some insights into a possible altered gene expression, and thereby indications of participation of some specific genes/proteins in the ovarian-specific pathophysiological events of PCOS. However, a drawback has been the use of either a whole ovary or random ovarian biopsies (95, 192, 193), with their heterogeneous population of cells and tissues. Furthermore, cells had been cultured *in vitro* (205, 207), rather than studied with regard to their expression in an *in vivo* situation.

In Paper I, we obtained cell/tissue material as part of PCOS infertility treatment, which allowed us to use fairly homogeneous tissue/cells. The findings would add to the knowledge of changes in the gene expression in PCOS ovaries and compare them with the expression in ovaries from regularly cycling women. In order to investigate the ovarian pathophysiology of PCOS we selected certain genes and two well-defined compartments (the central stroma and the GCs of the larger preovulatory follicles of the ovary), both thought to be central to the pathophysiology of PCOS. A key feature of PCOS is the arrested folliculogenesis and the consequent lack of follicular maturation (56, 57). Since the final maturation of follicles and the ovulatory process have been found to be intimately associated with inflammation (208-210), we concentrated our array approach to some central inflammatory mediators, and to other mediators that had been hypothesized by us and others to be of importance to the ovarian PCOS pathophysiology or to PCOS-associated diseases, such as OHSS. Thus, the aim of Paper I was to study the expression of these selected genes in two compartments (stroma and GC) of ovaries of PCOS women and to compare these expression profiles with the corresponding profiles of the same kind of tissue/cells from non-PCOS women and regularly cycling women.

Results

A number of the studied genes were found to be either over-expressed or under-expressed in the stroma. Dual specificity phosphatase 12 (DUSP12) and tissue factor pathway inhibitor 2 (TFPI2) were over-expressed. Five inflammation-related mediators (chemokine ligand 2 (CCL2), interleukin-1 receptor type 1 (IL1R1), interleukin-8 (IL8), nitric oxide synthase 2 (NOS2) and tissue inhibitor of metalloproteinase 1 (TIMP1)), the growth factor amphiregulin (AREG) and the inflammation-related transcription factor runt-related transcription factor 2 (RUNX2) were under-expressed in the ovaries of women with PCOS.

In GCs from the ovaries of women with PCOS, no under-expression of the studied genes was found but a number of genes were found to be over-expressed. These included two growth factors (bone morphogenetic protein 6 (BMP6) and DUSP12), the permeability-related aquaporin 3 (AQP3), the growth-arrest-related and DNA damage-inducible alpha (GADD45A) and five inflammation-related mediators (interleukin-1-beta (IL1B), IL8, leukemia inhibitory factor (LIF), NOS2 and prostaglandin-endoperoxide synthase 2 (PTGS2)), and the coagulation-related thromboplastin, also named coagulation factor 3 (F3) and thrombospondin 1 (THBS1). The specific changes in gene expression are summarized in Fig. 16.

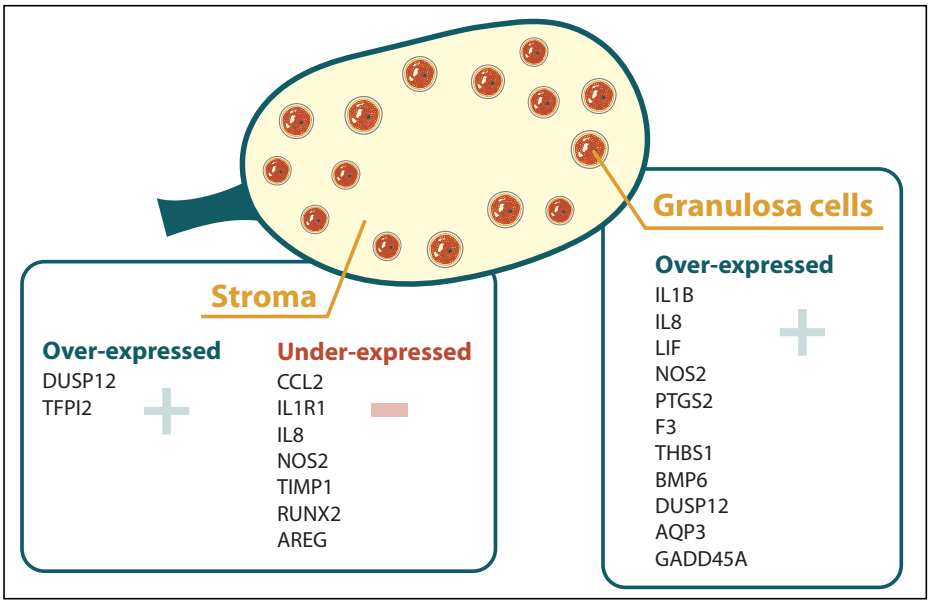


Fig 16 Schematic drawing illustrating the major findings of Paper I.

AREG=amphiregulin, AQP3=aquaporin 3, BMP6=bone morphogenic protein 6, CCL2=chemokine ligand 2, DUSP12=dual specificity phosphatase 12, F3=coagulation factor 3=thromboplastin, GADD45A=growth arrest and DNA damage-inducible alpha, IL1B=interleukin-1 beta, IL1R1=interleukin-1 receptor type 1, IL8=interleukin-8, LIF=leukemia inhibitory factor, NOS2=nitric oxide synthase 2, PTGS2=prostaglandin-endoperoxide synthase 2, RUNX2=runt-related transcription factor 2, THBS1=thrombospondin 1, TFPI2=tissue factor pathway inhibitor 2, TIMP1=tissue inhibitor of metalloproteinase 1. Plus (+) means over-expression of the specific gene in PCOS women compared with controls. Minus (-) means under-expression of the specific gene in PCOS women compared with controls.

Comments

The results of the present study are descriptive, rather than being the results of tests of function or ovarian-specific disease mechanisms in PCOS. Since little is known about possible intra-ovarian pathophysiological mechanisms in PCOS, a natural way is to start to screen the ovary for distinct patterns of under/over-expression of genes that may then point towards certain pathways or mechanisms that may be involved in the pathophysiology of the disease. Thus, the results obtained from these types of array studies can generate new hypotheses concerning disease mechanisms and thereby form the basis for complimentary studies that would specifically examine certain mechanisms/pathways and their roles in the pathophysiology of the ovaries of PCOS women.

The study was designed to investigate the central ovarian stroma and GCs of the antral follicles, as these were the tissues that could be obtained as a part of the treatment that these PCOS women underwent for their associated anovulation/infertility. Disease-specific changes in the genes studied could also occur in other cell compartments.
It is important to emphasize that the changes in the GCs should not be viewed as one phenomenon occurring strictly within these cells, but should be regarded as occurring in the microenvironment of the follicles where the GCs are located. The reason for this is the difficulty of retrieving a "clean" tissue sample for analysis without leukocytes, for instance, which are present in the follicle and will also be present in any follicular aspirate. The stroma is the microenvironment surrounding the follicles and interactions between all the cell types of the stroma are of importance; thus, it was adequate to study the cell compartment rather than separate cell populations.
It was a small and time-consuming learning curve to master the methods of obtaining the stroma samples from the PCOS women. However, it was even more difficult to obtain the GCs from the IVM patients, mainly because the patients were treated at another place.

Novel findings in this study were the existence of differences in the gene expression of inflammation-related genes in PCOS ovaries compared with the ovaries of healthy, regularly cycling women. Some of the inflammation-related genes that were found to be differently expressed in PCOS ovaries have previously been linked to ovarian events and may have implications for the arrested folliculogenesis (57, 96) (IL1B (209, 210), IL8 (209, 211), PTGS2 (212), CCL2 (213, 214)) in PCOS, the decrease in follicular atresia (215) (THBS1 (216)), the higher stromal blood flow (217), (NOS2 (218)) and the predisposition to OHSS (89) (IL1, IL8, (219) PTGS2 (220), CCL2 (221)). Some non-inflammatory genes were also differently regulated and these findings are further discussed in Paper I.
The exact mechanisms behind the findings in Paper I are unclear and the role of each differently expressed gene in these PCOS-pathophysiologic events in PCOS remains to be further elucidated. Possible ways to address these issues are to either use follicles/ovarian cells obtained from animal models of PCOS, such as the recently described new rat PCOS models (222), or to use cultured human ovarian cells from PCOS ovaries. In the latter case, GCs from PCOS patients undergoing IVM could be a natural source, but any cell culture using such cells would have to take place in a laboratory in close connection to the IVM lab. As pointed out earlier, it is important that the patients are well characterized with regard to their PCOS diagnosis and their hormonal aberrations, in order to minimize inter-individual variations within the PCOS group.

Reproductive hormone levels and anthropometry in postmenopausal women with polycystic ovary syndrome (PCOS): A 21-year follow-up study of women diagnosed with PCOS around 50 years ago and their age-matched controls (Paper II).

Changes in the adult blood levels of reproductive hormones in women in general starts already around the age of 20, with a decline in androgens (223). The decline in androgens continues until around 45 years of age (223). After that age, a minor decrease in total testosterone is described during the menopausal transition (224). The premenopausal drops observed in DHEAS (224) and androstenedione (223) continue after the menopause. Data on changes in SHBG are conflicting. The major and faster changes in reproductive hormones start approximately five years before the final menstrual period, when the declines in inhibins and estradiol start with a parallel and associated rise in FSH (225, 226). The decrease in inhibins and estradiol and the concomitant rise in FSH continue throughout the menopausal transition until the late postmenopausal period (225, 226).
It is well known that women after the menopause increase in weight and BMI, decrease slightly in height and redistribute fat from the gluteal to the abdominal region (227, 228).
Data on changes in reproductive hormones and anthropometry in PCOS women during the menopausal transition and later are sparse. In 1992, Dahlgren et al. (61) was the first to report on hormonal levels and anthropometry in perimenopausal PCOS women. Higher levels of testosterone, androstenedione, DHEAS, FAI and estrone were found in these women, compared with their age-matched controls. Furthermore, the PCOS population had a higher WHR, but lower levels of FSH and LH, and similar levels of SHBG and estradiol (61).
In 2000, Winters and co-workers published a cross-sectional study of a subset of PCOS women aged 47-57 years. They found lower levels of total testosterone and non-SHBG-bound testosterone, similar levels of SHBG and higher levels of FSH and LH in these perimenopausal PCOS women compared with younger PCOS women. In addition, in comparison with controls, the study showed higher levels of non-SHBG-bound testosterone levels and total testosterone levels (229). Higher androstenedione, total testosterone and DHEAS levels were also found in a recent study of exclusively postmenopausal PCOS women (mean age 55 years, n=20), compared to controls (230).
Paper II represents the first long-term follow-up study of women with PCOS and their randomly allocated age-matched controls, still similar with regard to BMI, concerning reproductive hormones and anthropometry during 21 years follow-up.

Results

In the study, 25 women with PCOS (mean age 70.4 years, range 61-79 years) and 68 controls were examined regarding reproductive hormones and anthropometry and they

also participated in a structured interview based on an identical questionnaire as in 1987. Only one woman was taking any kind of estrogens (PCOS, 1mg estriol orally).
BMI and WHR increased in women with PCOS as well as in controls during 21 years of follow-up. Body weight increased only in the control group while the PCOS women remained stable in weight during the follow-up period. The height decreased in both groups during follow-up. In 2008, the weight, height, BMI and WHR were similar in the PCOS women and the controls. In 2008, there was a similar prevalence of overweight and obesity in PCOS women and controls; 52% of the PCOS women and 62% of controls were overweight and 24% of PCOS and 18% of the controls were obese.
Estradiol, total testosterone, DHEAS and FAI decreased in the PCOS women and the controls during the 21-year follow-up. Androstenedione, SHBG and estrone remained unchanged in the PCOS women, but increased in the controls. FSH and prolactin levels were constant in both groups and LH levels increased in both PCOS and controls during the follow-up. TSH levels increased in both groups during the follow-up, whereas the proportion with positive anti-TPO was similar in both groups.
In 2008, FAI was higher, whereas SHBG and FSH levels were lower in the PCOS women than in the controls. The levels of total testosterone, androstenedione, DHEAS, estradiol, estrone, LH, prolactin and TSH were similar in both groups.
A re-evaluation of the data from 1987 showed positive correlations between FSH and age in both groups. A calculation of the age at menopause, based on when FSH levels would reach ≥50 U/L (the definition of menopause according to the laboratory used by us), predicted that the menopausal age would be 52.0 years for PCOS women and 46.5 years for controls.
However, the reported menopausal age was approximately the same in both groups.
In 2008; i.e. in the late postmenopausal age, women with PCOS had a higher prevalence of hirsutism (PCOS 64%, controls 9%), but had fewer climacteric symptoms and less hypothyroidism (PCOS 8%, controls 34%) than controls. A summary of the major findings of Paper II can be seen in Fig 17.

Comments

The findings of Paper II show, for the first time, that PCOS women also follow the age-related decrease in androgens, as previously described in non-PCOS women during the late postmenopausal age (223). Interestingly, the hyperandrogenicity persists in PCOS women of late postmenopausal age, although it seems to be less pronounced at this age compared with the fertile age (11, 60) and the perimenopausal period (61). The remaining hyperandrogenicity is in line with the sustained hirsutism in older PCOS women.

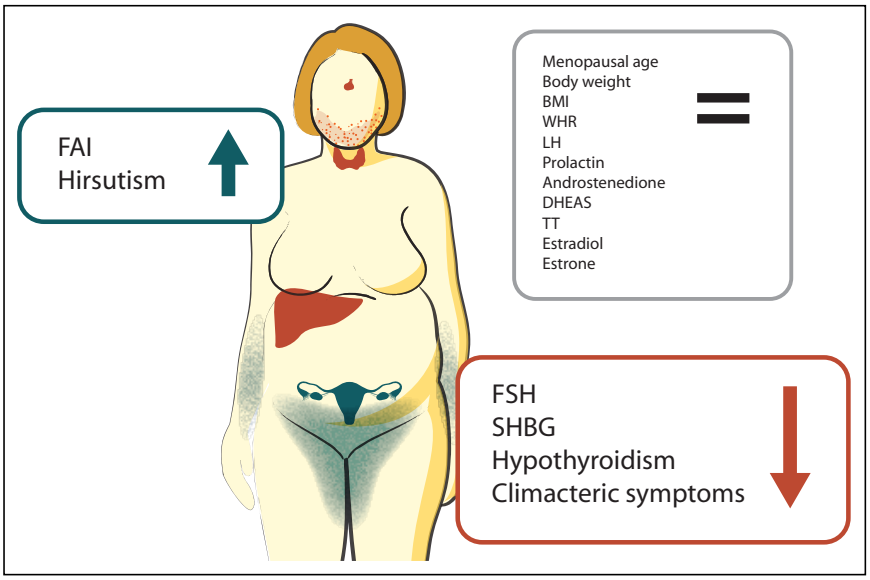


Fig.17 Schematic drawing illustrating the major findings of Paper II. ↑=increased, ↓=decreased, =similar, in PCOS women compared with controls.

The well-described age-dependent increase in body weight, BMI and WHR was seen in controls (227, 228). It is noteworthy that an increase in body weight was not seen among the PCOS women who remained stable in body weight during the follow-up period.

Another novel finding in Paper II was that the previously higher WHR associated with PCOS in the fertile and late fertile ages could not be detected during these late postmenopausal ages. One may speculate that this could influence the CVD risk factors and events in PCOS women, which are further studied in Paper III.

FSH levels were still lower in women with PCOS than in controls, also at these high ages. A mathematical model predicted a higher age of menopause among PCOS women, but this fact could not be detected among the study population. It has long been debated whether the fertile period of PCOS women are longer than that of normal women. There are some prediction models, based on AMH levels that have also indicated a later menopause among PCOS women (231). Prospective studies with serial hormonal measurements and examinations/questionnaires on this topic are needed to clarify this issue.

A novel finding of Paper II was the lower prevalence of hypothyroidism in PCOS women compared with controls. This finding needs to be verified in other prospective long-term studies before a firm conclusion can be drawn. Nevertheless, the existence of a lower risk of hypothyroidism among PCOS women may play a role in protecting PCOS women against CVD events in these high ages via beneficial effects on blood lipids (232).

Cardiovascular disease and risk factors in PCOS women of postmenopausal age: A 21-year controlled follow-up study (Paper III).

The knowledge of CVD risk factors in the general population has increased considerably during the last decades, mostly due to large prospective multicenter studies such as the INTERHEART (143, 153) and the WHO MONICA studies (182, 233, 234). The major non-modifiable CVD risk factors in women are age and a family history of premature CVD. The major modifiable risk factors are smoking, hypertension, dyslipidemia, obesity (in particular abdominal obesity), diabetes, psychosocial stress and a sedentary lifestyle, with the major risk factors for coronary heart disease in women being smoking, hypercholesterolemia and hypertension (233).

Multiple studies on young PCOS women and the few existing studies on perimenopausal PCOS women show an increased frequency of CVD risk factors. PCOS women also have an increased prevalence of the metabolic syndrome (138) which is associated with CVD (141) and all-cause mortality (142) in the general population. Due to this fact it has been speculated whether women with PCOS may have an elevated risk of CVD and mortality (61, 122, 185).

Dahlgren et al. indicated an approximately seven-fold increased risk of MI among PCOS women already in 1992 (183). This was based on a risk factor calculation model at that time. Since then, no conclusive evidence regarding the CVD risk in PCOS women has been presented.

Paper III represents the first long-term follow-up study of PCOS women and their age-matched controls (also similar in BMI) regarding CVD risk/events and mortality. The PCOS population studied was followed to high postmenopausal age when CVD events would be expected. The results of the analysis of blood samples in Paper III are based on the same PCOS women and controls as those who were examined in Paper II. Data on MI, stroke, hypertension, diabetes and cancer were obtained from the Hospital Discharge Registry and from the National Board of Health and Welfare Registry, with the latter providing data on mortality.

Results

Twenty-five women with PCOS (mean age 70.4, range 61-79 years) and 68 controls participated in all investigations (blood sampling, physical examination and the structured interview based on a questionnaire).

Morbidity data were obtained from 32 of 34 (94%) of the women with PCOS and from 95 of the 119 (80%) controls. Information on the cause of mortality was obtained in all the three deceased PCOS women and in all the 14 deceased controls.

Only one of the PCOS women was treated with estrogen (1mg estriol, orally) and none of the controls were taking any estrogen replacement. The use of lipid-lowering drugs was

similar in both PCOS women (24%) and controls (27%).
There were no differences in socioeconomic status, smoking, physical activity, age, BMI, body weight or WHR between the PCOS women and the controls.
The mean waist circumference increased among the PCOS women during the follow-up from 84 cm in 1987 to 91 cm in 2008. The mean waist circumference in controls increased from 80 cm to 89 cm. No differences in waist circumference or the proportion of overweight and obesity was seen between PCOS and controls in 1987 or 2008.
LDL and TG levels increased in both PCOS and controls during the 21-year follow-up. In 2008, the TG levels were still higher in the PCOS women than in the controls, but the higher HDL and lower LDL levels in the PCOS women detected in 1987 could no longer be seen. The total cholesterol levels were fairly constant during the study period and similar in both groups in 2008. Exclusion of the women with levothyroxine treatment did not change any levels of significance for any lipid-levels in the PCOS women and the controls.
In 2008, women with PCOS had a higher prevalence of hypertension (69%) than the controls (41%), as well as of hypertriglyceridemia. The levels of fibrinogen and PAI, insulin sensitivity (HOMA), the levels of insulin and the prevalence of diabetes were similar in PCOS women and controls.
The incidence of MI, stroke, death or age at death did not differ between PCOS women and controls at the end of the 21-years follow-up period. The calculated expected risk of death in the PCOS women from the time of wedge resection surgery, based on values from the general Swedish population, did not differ from the true incidence. In addition, the incidence of cancer was similar in the two groups. A summary of the major results of Paper III is presented in Fig. 18.

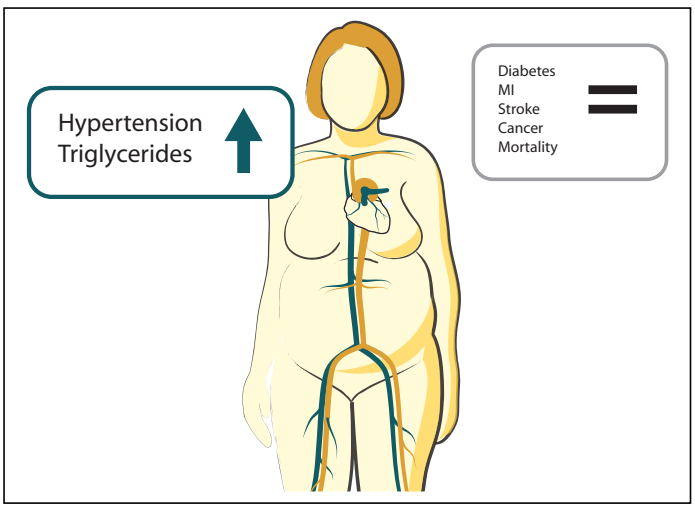


Fig. 18 Schematic drawing illustrating the major findings of Paper III. ↑=increased, **=**similar, in PCOS women compared with controls.

Comments

This is the first long-term follow-up study of postmenopausal PCOS women and controls regarding CVD risk factors and CVD end points. This subject has been heavily debated in the PCOS field during the past two decades, from the time when the proposed association between PCOS and increased MI morbidity/mortality was first suspected (183). The lack of prospective studies on this subject is probably due to the general problem with studies on PCOS women (*i.e.,* the lack of a well-defined PCOS population) and with prospective follow-up studies in particular where the problem of lost to follow-up is amplified, particularly by the advanced ages that need to be studied.

However, the sample size of the present study population was limited. Naturally, it would be an advantage to include larger study populations to achieve higher power. As a larger PCOS population sample was not available, and as we could not increase the participation rate further, we performed retrospective power calculations on deaths, CVD events and stroke. The results of these power calculations were predictable, as it showed that larger study populations are needed to achieve statistical power regarding these variables. For future research, we would suggest a prospective multinational multicenter study, but until such a study is performed, we may have to be satisfied with meta-analyses of smaller prospective studies.
Another aspect that further strengthens the results of the present study is that a calculation of the expected number of deaths in PCOS women shows no difference between PCOS women and the risk of death in the general Swedish population.

The results of Paper III could not be explained by any of the general confounding factors normally discussed in association with CVD, as there were no differences in socioeconomic status, smoking, physical activity, age, BMI, body weight or WHR between the PCOS women and the controls. Consequently no adjustment had to be performed concerning these factors.

Body composition, bone mineral density and fractures in late postmenopausal PCOS women – A long-term follow-up study (Paper IV)

Androgens are associated with increased muscle mass and increased BMD in general (107-109, 111). However, the data on bone quality in PCOS women are limited and conflicting, probably due to examinations of PCOS women with different inclusion criteria, different ages and different BMIs. Regarding age and BMI, the differences exist both between PCOS women and controls within the same study, but also between studies. There are no studies on bone quality or body composition in postmenopausal PCOS women.

We performed the first long-term follow-up of fractures in a well-characterized group of PCOS women. In 1992, BMD was measured by SPA and in 2008, DXA, the gold standard for measurement of bone quality, was used. In 2008, body composition (lean mass) was also determined in both the PCOS women and their age-matched controls. In addition, we investigated the fracture incidence, which is the most important end point regarding bone quality. The PCOS women and the controls were followed up to a high postmenopausal age when the increase in osteoporosis and fractures is known to occur. The group measured with SPA at baseline was a subgroup of the PCOS population examined by Dahlgren et al. in 1987 (61), and the groups examined by DXA in 2008 were subgroups of those participating in the studies of Paper II and III.

Results

At baseline 20, perimenopausal women (median age 52 years) with PCOS had a median BMD slightly above the reference level of the age-matched controls provided by the manufacturer for the SPA method.

In 2008, only one of the participants was using estrogen replacement therapy (PCOS; 1 mg estriol). Similar proportions of the PCOS women and the controls were taking calcium and/or vitamin D and/or bisphosphonates. The prevalence of smokers was similar in the two groups. The proportions in the two groups of a sedentary lifestyle and moderate and regular exercise were similar.

Twenty postmenopausal women with PCOS (median age 68.0 years) and 66 of the age-matched controls were examined by DXA in 2008. The lean mass and BMD were similar in the PCOS women and the controls, as was their BMI. The prevalence of osteoporosis, as defined by the WHO criteria, was also similar in PCOS women and controls in 2008. When the subgroup of hyperandrogenic PCOS women participating in the DXA examination was analyzed, this did not change any levels of significance regarding osteoporosis, or BMD in any measured body region.

There were no correlations between BMD and any sex hormone or FSH, LH or SHBG in the PCOS women. However, in the controls, total BMD was positively correlated to estradiol and FAI and negatively correlated to SHBG. The muscle mass of the postmenopausal PCOS women was similar to that of the controls. A novel finding was that the PCOS women had increased in hip circumference during 21 years, while the controls had not.

The PCOS women had a similar fracture incidence as compared with controls, including the incidence of osteoporotic fractures. The age at the different types of osteoporotic fractures was also similar to that in the controls. An illustration of the main findings of Paper IV is shown in Fig. 19.

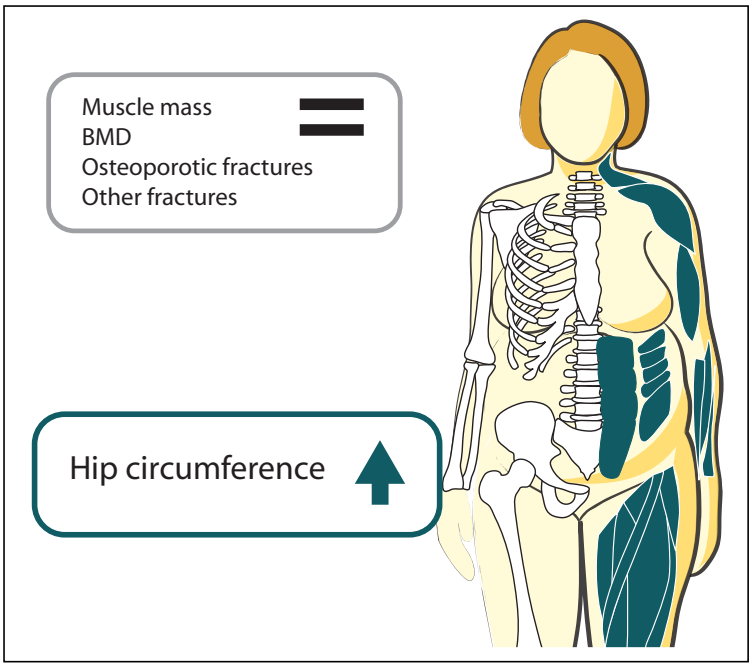


Fig. 19 Schematic drawing illustrating the major findings of Paper IV. ↑=increased in PCOS women during 21-years follow-up, =similar in PCOS women compared with controls in 2008.

Comments

This is the first study of lean mass and BMD in postmenopausal PCOS women and the first long-term follow-up study of fracture incidence in PCOS women. A strength of the study is the long time of follow-up, until late postmenopausal ages, when osteoporosis and fractures mainly occur.

It is important to stress that the groups compared in 2008 were similar with regard to age, BMI, percentage of smokers and the degree of physical activity. This is of importance since age, low BMI, smoking and a sedentary life style are known risk factors for osteoporosis and fractures (106).

At baseline, the perimenopausal PCOS women seemed to have a BMD above that of the age-matched reference group. This result is in line with the hypothesis of this study and could be explained by the hyperandrogenicity of the PCOS women. However, it is noteworthy that the BMD (and lean mass) in the late postmenopausal ages in the PCOS women was not higher than in their controls, despite their persisting hyperandrogenism (Paper II).

The fracture incidence was also similar; however, the sample size was limited.

Another novel finding was the increase in hip circumference in aging PCOS women, which is in contrast with the normal aging process in women, with an increase in waist circumference rather than the hip circumference (228). This was also verified when the data of the total study population in Paper III was analyzed. This may contribute to the lack of further detoriation of CVD risk factors and the lack of more CVD events in the aging, late postmenopausal PCOS women compared with women in the general population (paper III).

Discussion

In the Discussion section I have chosen to discuss some specific results of the thesis, which merit discussion in a broader context. Other results are discussed to some extent in the Results and Comments section and/or in the Papers.

Hyperandrogenism

PCOS women maintained hyperandrogenism throughout the senium. However, a decrease in androgens also occured in women with PCOS with increasing age. This is in line with studies in the general population, which report that levels of DHEAS, total testosterone and androstenedione start to decrease already at the age of 20 (223), with a continuous decline until 45 years of age (223). Thereafter, a minor change in total testosterone is described during the postmenopausal transition (224, 235), in contrast with the continuous drop in DHEAS (224) and androstenedione (223). DHEAS has been shown to decrease continuously at the same speed until 75 years of age (223), but for androstenedione and total testosterone, a regression model suggests a late postmenopausal rise starting around the age of 71 years for androstenedione and around 62 years for total testosterone (223, 236). Also, some other smaller cross-sectional studies of testosterone in non-PCOS women have shown stable age-related changes in testosterone, but some studies showed increasing testosterone levels with age (236-238).

A decrease with age in total testosterone and non-SHBG-bound testosterone, compared with younger PCOS women, has also been shown in a cross-sectional study of a subset of 21 PCOS women aged 47-57 years. (229). In contrast with the results of that study, a recent cross-sectional report of PCOS women showed stable levels of basal androstenedione, DHEAS and testosterone when premenopausal and postmenopausal PCOS women (aged up to 59 years) were compared (239). The contrast between the results may be explained by small sample sizes in both studies and/or by the assumption that the decline in androgens may be steeper after the perimenopausal period and that this sharper drop was not caught in these previous studies.

Data seem to be conflicting regarding changes in SHBG with age in the general population. One of the larger cross-sectional studies showed stable levels until ~65 years of age, followed by a minimal decrease until 75 years of age (223). Another cross-sectional study in an American Caucasian population showed increased SHBG with age (236). Burger et al. showed a decrease in SHBG in their prospective longitudinal study on Australian women up to 62 years of age, and also a 4% lower SHBG for each kg/m² of increase in BMI (224). This finding is in concordance with the fact that weight gain decreases SHBG and with the secular trend of a weight gain in aging women (182).

Decreasing SHBG levels with time is also supported by a recent large study (n=629) showing an age-related decline in women aged up to 59 years of age (235).
Constant levels of SHBG were seen in the cross-sectional study of PCOS women by Winters et al., comparing 42-57-year-old PCOS women with younger PCOS women (229). This was in line with the findings of another small cross-sectional study comparing premenopausal- and postmenopausal PCOS women aged up to 59 years (240). The stable SHBG level in the PCOS women of the present study (Paper II) is probably explained by the constant body weight in the group with PCOS women during the 21-year follow-up. However, the increase in SHBG in controls was unexpected as they increased in weight during the follow-up, which should result in decreased SHBG levels. This increase could possibly, in part, be explained by the rise in estrone levels with time in controls, which may stimulate the production of SHBG to some extent.
The increase in androstenedione among controls (Paper II) is in line with the calculated suggested late postmenopausal increase in the general female population (223). The stable levels of androstenedione in PCOS women (Paper II) confirm the results of the cross-sectional study by Puurunen et al. (239), who compared the levels in early postmenopausal women (up to the age of 59 years) with the levels in premenopausal PCOS women. Our findings suggest further continued stable androstenedione levels into the late postmenopausal period in PCOS women.
The decline in testosterone during the postmenopausal period of PCOS women may be explained by a relatively decreased activity of the enzyme that converts androstenedione to testosterone, which have been speculated to have a higher efficacy in premenopausal PCOS women compared with non-PCOS controls (43, 241).
The decreases in the production of total testosterone, androstenedione and DHEAS in PCOS women compared with non-PCOS women seem to be late postmenopausal events, as it has been shown in two studies that basal testosterone, androstenedione and DHEAS levels were higher in postmenopausal PCOS populations with a maximum age of 62 years and a mean age of 55 years, respectively, compared with non-PCOS controls (230, 239). Furthermore, in the study of PCOS women aged up to 57 years by Winters et al. (229), the higher levels of total testosterone in the PCOS women remained compared with the controls. Accordingly, the findings of Paper II in combination with other studies suggest that the hyperandrogenism partly resolves in PCOS women, starting before the menopause (229), is stable during the menopausal transition (239) and continues until late into the postmenopausal period (Paper II).
Despite a decrease in FAI with time in late postmenopausal PCOS women, they still had higher FAI than controls. This persistent elevation in FAI in elderly PCOS women is most likely the cause of the remaining higher prevalence of hirsutism in the older PCOS women, as found in Paper II.

A consequence of the decline in the hyperandrogenism in elderly PCOS women may be a decrease in insulin resistance/hyperinsulinemia, as there is a known strong association between these factors (63, 242). This relationship was also confirmed in Paper III by the similar insulin levels and HOMA indices in PCOS women and controls, and also by the prevalence of diabetes no longer being higher among late postmenopausal PCOS women than among the controls. However, a prerequisite for this is maintained SHBG levels and, thus no increase in weight (224), which would cause an increase in free bioavailable androgens. This means that the decrease in androgens may disrupt the vicious circle in PCOS and reverse the metabolic aberrations associated with pre/perimenopausal PCOS, resulting in a reduced prevalence of CVD risk factors and the consequent risk of CVD events, which is verified in Paper III.

The decrease in the androgenicity in the PCOS women with age may also explain why late postmenopausal PCOS women did not have a higher BMD than controls (Paper IV), as androgens are known to exert anabolic effects on bone (107, 108). Furthermore, the decrease in androgens during the postmenopausal period and the subsequent decrease in insulin resistance/hyperinsulinemia could have a negative effect on ovarian IGF-1, which, among several actions, stimulates ovarian cell proliferation (49). This could affect the size of PCOS ovaries during the postmenopausal period.

FSH, ovarian reserve and menopausal age

Late postmenopausal PCOS women had lower levels of FSH compared with controls (Paper II). This result persisted also when the women who had undergone oophorectomy in previous years were excluded. Lower FSH levels in PCOS women compared with controls are in line with the findings in the same study population 21 years ago, when the 33 PCOS women were aged 40-59 years old (61). All the PCOS women who were included in the original study had undergone ovarian wedge resection at a mean age of ~25 years. Ovarian wedge resection involves excision of a considerable part of the ovary, and this would normally lead to a decrease in the ovarian reserve. Secondary to this, lower levels of inhibin and estradiol would be anticipated, as well as an increase in FSH. Thus, despite the ovarian surgery performed on PCOS women they had still lower FSH levels than controls. This has also been shown by another research group, which reported lower FSH levels in PCOS women compared with their age-matched controls, in women around 25-30 years of age (243). In another study, the authors showed lower FSH levels in a group of PCOS women with a mean age of 43.2 years (range: 37-49 years), compared with controls (244). The cause of the lower FSH levels in PCOS women during the fertile period is not clear, but it may be related to the increased production of inhibin B (245) and/or AMH (246) by the increased number of small antral follicles in ovaries of PCOS patients (54, 96).

A number of previous studies of PCOS women of fertile age lend support to the theory of an increased ovarian reserve in PCOS women in comparison with their age-matched controls. Thus, a higher antral follicle count, greater ovarian volume (187, 244) and higher AMH levels (54, 244, 246) have been demonstrated. The results of the present study, with lower FSH levels at a stage in life when the ovaries are depleted of follicles, can, of course, not be explained by the same mechanisms as those in the play during fertile ages. As the ovaries are inactive with regard to the production of inhibin and estradiol late in life, this is most likely a direct effect at the pituitary level. It may well be that the pituitary has been tuned during the fertile period to produce less FSH and this continues also at a stage when there is no negative feedback from the ovaries.
In the light of the findings regarding FSH, antral follicle count and AMH described above, one would expect that women with PCOS would have a prolonged fertile life, due to an increase in the ovarian reserve. This assumption is also based on the proposed larger follicle pool in PCOS women from birth, which was based on the finding of a greatly increased density of small preantral follicles in a study of ovarian biopsies from PCOS and non-PCOS women (96).

In the present thesis, the PCOS women reported a similar age at menopause as controls, a finding in line with the results of the large retrospective study by Wild et al. (247). However, due to the finding of lower FSH levels in PCOS women and the knowledge that a greater proportion of the PCOS women in 1987 (21% *vs.* 7%) were hysterectomized (61), with the age at hysterectomy often being self-reported as the menopausal age (our unpublished data), we calculated the age at menopause on the basis on FSH levels in 1987 (Paper II). According to this model, PCOS women should reach menopause 5.5 years later, in agreement with the findings of another study based on AMH levels (231). A somewhat, although insignificantly, later calculated age at menopause was also shown in another study, also based on AMH levels (248).

Our results should be interpreted in the light of the FSH being a well-established menopausal marker, which is also considered today by the STRAW (STages of Reproductive Ageing Workshop) to be the most suitable available biomarker for indication of the onset of the late reproductive age (249, 250). Other biomarkers, such as AMH and inhibin B, appear to change earlier than the FSH level during the reproductive aging process (97). The present findings need to be validated in other prospective studies using the FSH level as a marker. These studies should also compare the actual age of hysterectomy in PCOS women with the reported age of menopause. Populations should be seen at yearly visits, which should preferably also include transvaginal ultrasound to ascertain hysterectomy status or evaluation of the endometrial thickness and/or ovarian

volume as complimentary measures to determine the menopausal age. Delayed ovarian aging and a delay in the menopausal transition with a de facto later menopausal age in women with PCOS may be one mechanism behind a possible, so far unknown, protective factor of CVD in these women. A de facto later menopausal age among women with PCOS could also explain the tendency towards a BMD above the reference levels found in the perimenopausal PCOS women, as they would be exposed to estrogens for some additional years.

Body composition

It is well known that women increase in weight and BMI after the menopause and redistribute fat from the lower to the upper body (227, 228), resulting in increased abdominal obesity measured as a larger waist circumference and/or WHR. Furthermore, women of postmenopausal age usually decrease in height (228).

Premenopausal PCOS women usually have increased abdominal fat distribution independently of BMI (117, 133, 155). This has also been shown to persist among perimenopausal PCOS women, for whom Dahlgren et al. showed a higher WHR in the original study population of 33 PCOS women aged 40-59 years (61). This is the cohort on which Paper II-IV are based. Also, Wild et al. (185) showed a higher BMI and higher WHR in PCOS women with a mean age of 57 years (although with a very large range of 38-98 years) compared with controls. Thus, little is known regarding anthropometry in postmenopausal PCOS women.

Both the PCOS women and the controls in this thesis increased in BMI, WHR (Paper II) and waist circumference over time (Paper III), while a decrease in height (Paper II) was seen in both groups. Thus, PCOS women follow the trends of the general population, but importantly, the PCOS women did not increase in weight in contrast to the controls. Furthermore, the PCOS women increased in hip circumference, whereas the hip circumference of the controls remained constant (Paper IV). Thus, the previously higher WHR in 1987 (61) had disappeared over time and similar WHRs were seen in PCOS women and controls at the 21-year follow-up.

Taken together, late postmenopausal PCOS women seem to differ from controls with regard to the “normal” changes in anthropometry, causing them to lose their previously increased abdominal fat compared with controls. The control women appeared to “catch up" with age with regard to their abdominal fat distribution, which is a metabolic disadvantage. The relative decrease in abdominal fat among postmenopausal women with

PCOS is a novel finding and important in view of the associated CVD risk factors. Abdominal fat has been associated with dyslipidemia, insulin resistance, T2DM, hypertension, CVD and mortality (143-146, 148). Thus, the decrease in the abdominal fat distribution in elderly PCOS women might lower their prevalence of CVD risk factors and CVD events and mortality to the same level as that of the general female population.

In 2008, the PCOS women participating in the studies of Paper II-IV had a mean BMI of 27 kg/m², with the controls being similar in BMI. The mean BMIs of other cohorts of PCOS women/controls (mean age ~50-60 years) included in other studies are commonly higher (~28-31 kg/m²) (184, 229). Also, in a Scandinavian perspective our PCOS study population was relatively lean, exemplified by a recent study by Hudecova et al. (Uppsala, Sweden), where PCOS women aged 37-49 years had a mean BMI of 28 kg/m² (244) and by a recent Finnish study by Puurunen et al. in which the exclusively postmenopausal PCOS group had a mean BMI of 34 kg/m² (mean age 55.6 years) (240). However, the BMI, weight, WHR and height in our study performed in 2008 were in line with findings in other randomly selected postmenopausal non-PCOS women originating in the same urban area (182, 201). Thus, the results of Paper II-IV may be unique to Sweden and, in particular, the urban area of Gothenburg in many aspects.

The leaner BMI of the PCOS women in the present study may mean a lowered risk of CVD. Thus, the lower BMI of our PCOS population may, at least in part, explain the similar prevalence of dyslipidemia (except for the higher levels of triglycerides in PCOS), insulin resistance (assessed by HOMA) and diabetes, the similar levels of fibrinogen and PAI-I in PCOS women and controls, and the similar prevalence of MI, stroke and age at death (Paper III).

CVD risk factors and CVD

The PCOS women, with a mean age just above 70 years, had a higher prevalence of hypertension and higher triglyceride levels than the controls, whereas the levels of other blood lipids (total cholesterol, LDL, HDL, ApoA, ApoB, ApoB/ApoA1), insulin, glucose, fibrinogen and PAI as well as HOMA were similar to those of the controls. In the study by Dahlgren et al. higher levels of triglycerides and HDL were found in PCOS women than in controls, as well as a higher prevalence of hypertension and diabetes at the mean age of ~ 50 years, at baseline in 1987 (61). Wild et al. (185) only found a higher prevalence of hypercholesterolemia after adjustment for BMI in PCOS women with a mean age of ~57

years, compared with controls. Thus, the discrepancy in the number of risk factors in PCOS women compared with controls seems to decrease over time in elderly postmenopausal PCOS women. The similar prevalence of diabetes and insulin resistance in the PCOS women and controls in Paper III is in line with the results of a Finnish study by Puurunen et al. (240). They found persistent insulin resistance (based on oral glucose tolerance test) in a subgroup of 11 postmenopausal PCOS women (aged up to 59 years). However, after adjustment for BMI, the authors concluded that the difference in glucose metabolism did not persist (240). It will of course be of great interest to look at even older cohorts of similarly well-defined PCOS women to see if the CVD risk factor pattern will be more like the pattern in the general femal population at ages above 75 years.

The major findings in this thesis (Paper III) were that women with PCOS did suffer from more MI, stroke, or death caused by CVD than controls, when followed up to a mean age of ~70 years. These findings were surprising, considering the long exposure time of increased CVD risk factors in PCOS women during the young, perimenopausal and postmenopausal (Paper III) ages. Also, in 1987 Dahlgren and co-workers (183) had predicted a sevenfold increased risk of MI in the PCOS population based on a metabolic risk factor model applied to the same PCOS women as in Paper III. In addition, a recent (and the only) meta-analysis on the risk of (non) fatal coronary heart disease or stroke in PCOS women indicated a BMI-adjusted relative CVD risk of 1.55 in PCOS women (186). However, the finding of no increased mortality in PCOS women in Paper III confirms the results of the register studies by Pierpoint et al. (122) and by Wild et al. (185), where PCOS women were found not to have a higher than average all-cause mortality or mortality from CVD. Nevertheless, it should be pointed out that Wild and co-workers (185) found a slight increase in the OR for cerebrovascular disease. Hypertension is one of the strongest risk factors for stroke (158) and hypertension was one of the persisting risk factors in the elderly PCOS women (Paper III). Thus, it cannot be excluded that elderly PCOS women may have an increased risk of stroke, which would become evident at even older ages than those studied in the present thesis.

The decrease over time in CVD risk factors in elderly PCOS women compared with controls may be explained in part by the common secular trends in society, with a decrease in smoking, increased physical activity, intake of healthier food and thereby decreasing cholesterol levels (233, 234). These lifestyle factors have effects on the incidence of CVD events and the mortality rate. However, the controls caught up with the PCOS women with regard to body weight and abdominal fat distribution (Paper II). The PCOS women in our study may have been protected by the higher HDL levels seen in 1987 (175) (Paper III).

Hyperandrogenism in younger PCOS women have been reported to be associated with a worsening of cardiovascular risk factors (dyslipidemia, abdominal obesity, hyperinsulinemia) via insulin resistance (126, 132, 161). Shaw et al (178) reported that CVD events were associated with higher levels of all the androgens in PCOS women with a mean age of 62 years. They used a risk-adjusted predictive model, controlling for an array of traditional cardiac risk factors, and based on their results, they suggested that the prolonged hyperandrogenism in PCOS may be one mechanism responsible for the higher cardiac risk found in the PCOS women in their study. Thus, the fact that CVD events and CVD-related mortality do not occur more often in the PCOS population (Paper III) than in the general female population, despite the apparent presence of risk factors, might also be explained by the partly resolving hyperandrogenism during the postmenopausal years (Paper II).

The sevenfold increased risk of MI predicted by Dahlgren et al. (183) could not be verified in Paper III. The risk factor model at that time was based on the independent risk factors of hypertension, diabetes, serum triglycerides and WHR multiplied by a function of age. The most powerful risk factor was WHR (factor 7.45) and the second strongest was hypertension (factor 1.75). Thus, the reason why we could not find the predicted increased risk of MI may have been due to the fact that the WHR difference between PCOS women and controls had disappeared at follow-up and/or by the fact that WHR has been proven not to be as strong risk factor as it was believed in 1987.

In the meta-analysis on the risk of (non) fatal coronary heart disease and stroke in PCOS women, by de Groot and co-workers, a total number of 1340 papers were identified as potential sources at the start. Only five studies met the inclusion criteria and, of those, only two were prospective (178, 251). In addition, two of the studies (184, 251) did not use hard clinical outcomes of acute MI, but included quite a large group of "probable" cases of non-fatal MI/stroke. Furthermore, the highest ranked study by Shaw et al. (178) may have been selection-biased as they examined a group of women who had undergone a clinically indicated coronary angiogram for suspected ischemia. These women were later asked about a previous history of irregular menses (104 women out of 390 enrolled in the angiogram study were retrospectively diagnosed with PCOS). Thus, the retrospective design with the possible recall bias may influence the results. It should also be mentioned that four of the five included studies were performed in countries with a relatively high BMI (average mean BMI >27.5 kg/m²; average mean age ~60 years) namely the US (178, 251), the UK (185) and the Czech republic (184). Only one study was from a country with a relatively low average BMI, Norway (252), and that study was the only one which showed a relative risk of less than 1 (0.92). Thus, all countries except for Norway were

countries that had a relatively higher average BMI than Sweden, which is also mirrored by the BMI of PCOS populations in other countries (2-4).

One might speculate that due to the relatively leaner PCOS population in Gothenburg Sweden, the results of this thesis might not be applicable to PCOS populations with higher BMIs. Despite the small sample size, these results may instead prove that CVD events could be avoided, even in PCOS women with a higher prevalence of CVD risk factors, if the BMI is kept relatively low.

Hypothyroidism

There are a few studies regarding thyroid function in PCOS women of reproductive ages, and the data seem inconsistent. Some studies report a higher prevalence of increased TSH levels (253) or associations of hypothyroidism and PCOS (254). However, a very recent textbook on PCOS from Royal College of Obstetricians and Gynaecologists reports the same incidence of hypothyroidism (~ 5%) in women of fertile age with hyperandrogenism as in non-PCOS women of reproductive age (24).

Studies regarding hypothyroidism in postmenopausal PCOS women are lacking. The present data (Paper II) suggests a decreased predisposition for hypothyroidism in elderly postmenopausal PCOS women (8%) compared with controls (34%). The lower prevalence in the PCOS women persisted when a comparison with the larger random population sample of women from the WHO MONICA study in Gothenburg was made (182). In the larger WHO MONICA population of women of the same age, 18% were found to have hypothyroidism in 2008 (Landin-Wilhelmsen, personal communication). A similar proportion of PCOS women (19%) and controls (20%) had elevated positive anti-TPO levels in Paper II, suggesting that the hypothyroidism was not of autoimmune origin.
It could be speculated that hypothyroidism would be more common in PCOS women, as hypothyroidism is associated with irregular periods, increased BMI and insulin resistance in non-PCOS women (255); features that are common in PCOS. On the other hand, it may further be speculated that the decreased prevalence of hypothyroidism in the elderly PCOS women may, at least in part, be explained by the persisting hyperandrogenicity (Paper II). Hypothyroidism is less frequent in men and a protective effect of androgens on thyroid function is indicated by hypothyroidism in men being associated with lower testosterone levels (256). However, TSH and free thyroxin were unaffected by ethinyl estradiol and antiandrogen treatment in PCOS women (257).

If this lower prevalence of hypothyroidism among PCOS patients is confirmed by other studies, it may have important implications for lowering the risk of CVD in PCOS women. This is based on a possible positive association between hypothyroidism and CVD risk factors (258, 259) and CVD (260-262), and the link seems to be the association between hypothyroidism and dyslipidemia (232) .

Muscle mass, bone mineral density and fractures

The BMD in the PCOS women with a median age of 52 years was above the age-matched reference levels of the manufacturer. When re-investigating the women at a late postmenopausal age (median age 68 years) using DXA, the BMD and lean mass were similar to that of the controls. The BMD above the reference level in women with PCOS during the perimenopausal age may be explained by the hyperandrogenicity of the PCOS women, *i.e.,* the proved anabolic effects of androgens on bone (107, 108). However, the higher z-score in perimenopausal ages did not persist until the late postmenopausal ages, despite of the remaining hyperandrogenism (Paper II). The levels of androgens did, however, decrease, also in the PCOS women (Paper II), and there may be a threshold for the androgens where they are no longer able to have an anabolic effect on bone. Besides, and probably more importantly, the almost undetectable levels of estradiol have to be considered at these high menopausal ages (Paper II). The beneficial effect of estradiol on bone is well-known (110, 263). Taken together, this raises important questions regarding bone physiology at this postmenopausal age and it may be that the bone in PCOS women is more similar to bone in men where estradiol is needed for the androgens to exert an anabolic effect on bone (107, 108). This theory is strengthened by the correlation calculations of total BMD and sex hormones in the present study, as inverse slopes were observed in the correlation coefficients of BMD versus estradiol in PCOS compared with controls, and as no correlations were found between BMD and androgens in PCOS women, but were present in the controls. In addition, long-lasting exposure to androgens, as is the case for postmenopausal PCOS women (Paper II and (230), may inhibit the proliferation of osteoblasts (108), which may explain why postmenopausal PCOS women did not have higher BMD than controls.

Whatever the mechanisms, it can be concluded that PCOS women decrease in bone mass after the menopausal transition during aging, as women in general (263).

One may speculate about the existence of a pattern of age-related changes in BMD in PCOS women. This statement is based on previous findings of a similar BMD (assessed by DXA) in PCOS women and controls of adolescent (115) and younger ages (similar mean age 24 years in the two studies by Adami et al. and by Carmina et al.)

(114, 118). Furthermore, at the mean age of 28 years, the BMD has been seen to be higher in the upper limbs of PCOS women compared with age-matched and weight-matched controls (116). This was also confirmed in a recent study of PCOS women with a mean age of 28 years, in whom higher volumetric cortical density, as assessed by peripheral quantitative computed tomography (pQCT), was found among PCOS women compared with their age-matched controls (103).
The same age-related changes might be assumed to be present in PCOS women regarding lean mass. A lower lean mass has been found in adolescents (264) and girls with PCOS in their early twenties (117), and increased lean mass has been shown in women with PCOS at a mean age of 24 years (118) compared with controls. The decrease in lean mass after the menopause may be explained by the declining androgens levels after the menopause (Paper IV).

Similar fracture incidences, including the incidence of osteoporotic fractures, were seen in the postmenopausal groups (Paper IV). This is in line with the other findings of similar BMD and similar muscle mass in PCOS women compared with controls, as an increased muscle mass is known to be positively associated with BMD (108), and an increased BMD leads to a lower risk of fractures (106). The similar fracture incidence is supported by the similar total cholesterol levels in the PCOS women and the controls, both at baseline and at follow-up. This is based on serum total cholesterol having recently been found to be an independent risk factor for fractures in the main cohort of the random WHO MONICA population followed during the same time period of more than 20 years (201).
The lack of a difference in BMD and fractures between PCOS women and controls is in line with the similar incidence of mortality in the two groups (Paper III), as fractures are associated with increased mortality (265).

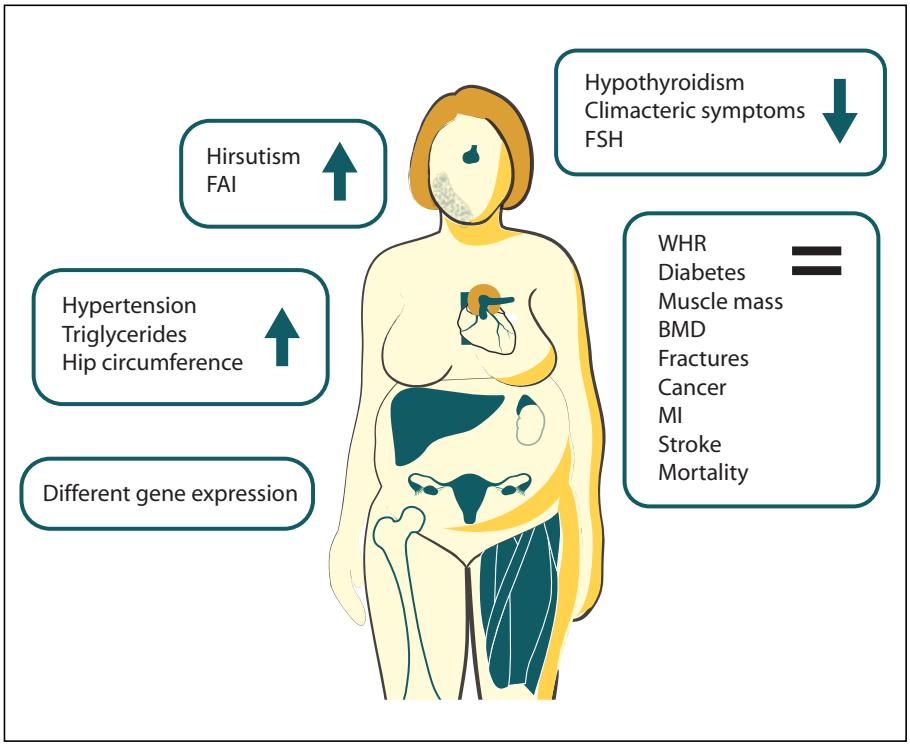
Limitations and strengths

The major limitation of all the papers included in this thesis is the small sample size. Furthermore, in the study of gene expression, the PCOS women of the GC group (Paper I) could have been better characterized with regard to their biochemical hyperandrogenism. In paper II-IV, RIA methods were used for the analysis of sex hormones. These methods were the methods of choice in 1987, and in 2008 we aimed for similar methods as those used in 1987. Further, BMD was assessed using SPA in 1992 and DXA in 2008, as DXA was not available in 1992. Questionnaires were used to obtain fracture data. Validation by registry data would have strengthened the study.

The strengths of the studies are the unique tissue samples from the two different compartments of the ovary from PCOS women and controls (Paper I). Paper II-IV represent the first prospective long-term follow-up study of PCOS women and controls. The long follow-up time (21 years) and the follow-up into the late postmenopausal ages are strengths. In addition, there were fairly high participation rates (average participation rates for the subgroups studied were ~78% among PCOS women and ~64% among controls), given that 21 years had passed between the two study occasions. Furthermore, the PCOS and control groups remained age-matched despite the uncontrolled drop-out rate. It is also noteworthy that there were no differences between the groups with regard to socioeconomic status, BMI, weight, smoking and physical activity in 2008. These are all factors that it would otherwise have been necessary to adjust for. Besides, the cohorts were treatment-naive to estrogens and similar proportions of PCOS women and controls were using lipid-lowering and anti-osteoporotic agents.

Polycystic ovary syndrome

Ovarian pathophysiology and consequences after the menopause



↑=increased, ↓=decreased, =similar, in PCOS women compared with controls.

General conclusions

- Differences existed between women with PCOS and controls with regard to the ovarian expression of certain genes (mainly of inflammatory origin) of importance in the ovarian pathophysiology of PCOS. This may be of importance for the arrested folliculogenesis and for the predisposition of ovarian hyperstimulation syndrome (OHSS) in PCOS women.

- Postmenopausal women with PCOS had persistent hyperandrogenism and hirsutism compared with controls.

- FSH levels remained lower in women with PCOS than in controls, despite the similar reported menopausal age.

- The elderly PCOS women displayed a positive shift towards a more gluteo-femoral fat distribution, in contrast with the controls from the general female population who gained weight with a preponderance of abdominal fat distribution.

- The late postmenopausal women with PCOS had a lower prevalence of hypothyroidism than controls.

- Late postmenopausal PCOS women had a higher prevalence of hypertension and hypertriglyceridemia.

- Women with PCOS had similar muscle mass and BMD, and a similar prevalence of fractures, cancer, MI, stroke and mortality as controls.

- PCOS women should be recommended to keep their BMIs relatively normal and they should be treated as women in the general population regarding prevention of CVD.

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