

Polycystic ovary syndrome

Studies of metabolic and ovarian disturbances
and effects of physical exercise
and electro-acupuncture

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2010



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Cover illustration by Joen Wetterholm

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Printed by Geson Hylte Tryck Gothenburg, Sweden, 2010

ISBN 978-91-628-7896-2

<http://hdl.handle.net/2077/21477>

Till min familj

ABSTRACT

Polycystic ovary syndrome (PCOS) is the most common endocrine abnormality in premenopausal women. The syndrome is characterized by hyperandrogenism, ovulatory dysfunction and polycystic ovarian (PCO) morphology. Metabolic disturbances, such as insulin resistance and obesity, are also associated with PCOS. Despite extensive research, the etiology and pathophysiological mechanisms of PCOS and related metabolic disturbances are largely unknown. The clinical management of PCOS is multifaceted but often unsatisfactory.

The main aims of this thesis were 1) to develop new rat PCOS models displaying ovarian and/or metabolic abnormalities, and to evaluate the effects of low-frequency (2 Hz) electro-acupuncture (EA) and physical exercise in the most complete of these models, and 2) to characterize the adipose tissue of women with PCOS (normal weight/overweight/obese) in terms of distribution, cellularity, lipid metabolism, release of certain adipokines and macrophage density, and to identify factors among these characteristics and serum sex steroids that are associated with insulin sensitivity in these women.

Female rats were continuously exposed either to the aromatase inhibitor letrozole or the nonaromatizable androgen dihydrotestosterone (DHT), starting before puberty, to induce a hyperandrogenic state. All rats exposed to letrozole became anovulatory and developed PCO morphology with structural changes strikingly similar to those in human PCOS, but without the metabolic abnormalities. Rats exposed to DHT displayed alterations in ovarian morphology and function, as well as metabolic abnormalities that included adiposity, enlarged adipocytes and insulin resistance in adulthood.

EA and exercise improved both insulin resistance and ovarian morphology in rats with DHT-induced PCOS. These results indicate that both interventions break, at least partly, the vicious circle of androgen excess, insulin resistance and ovarian dysfunction in PCOS. Both EA and exercise also partly restored altered adipose tissue gene expression related to insulin resistance, obesity, inflammation and high sympathetic activity, suggesting that exercise and EA may both influence regulation of adipose tissue metabolism/production and sympathetic activity. Interestingly, in contrast to exercise, EA exerted its beneficial effects without influencing adiposity or adipose tissue cellularity.

Compared to controls pair-matched by age and body mass index (BMI), women with PCOS had larger abdominal subcutaneous adipocytes, lower plasma adiponectin, and

lower LPL activity (borderline significant). There were no differences in anthropometrical variables or in abdominal volumes of total, subcutaneous and visceral adipose tissue, as determined by MRI, between the groups. Women with PCOS also had lower insulin sensitivity, higher serum levels of testosterone, free testosterone and free estradiol as well as lower serum levels of sex hormone binding globulin. Multiple linear regression analysis revealed that adipocyte size, circulating adiponectin and waist circumference, but not circulating sex steroids, were the factors strongest associated with insulin sensitivity in women with PCOS.

In conclusion, androgens are likely to play a central role in the pathogenesis of PCOS. Our rat models of PCOS highlight the close relationship between androgen excess and the development of ovarian and/or metabolic disturbances typical of this syndrome. Women with PCOS display hyperandrogenemia, insulin resistance and adipose tissue abnormalities, although their adipose tissue distribution and abdominal volumes are indistinguishable from age/BMI-matched controls. The adipose tissue abnormalities in PCOS — enlarged adipocyte size and low circulating adiponectin — together with a large waistline, rather than the hyperandrogenemia, seem to be central factors in the development/maintenance of insulin resistance in these women. EA and exercise may both represent valuable non-pharmacological treatment alternatives in PCOS, with the potential to improve both ovarian dysfunction and metabolic disturbances.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Polycystiskt ovariesyndrom (PCOS) drabbar ungefär var tionde kvinna och är därmed den vanligaste hormonella rubbningen hos kvinnor i fertil ålder. Dessa kvinnor har höga nivåer av manligt könshormon (androgener). Syndromet ger bland annat ökad kroppsbehåring, akne och oregelbunden eller utebliven menstruation samt nedsatt fertilitet. Benämningen ”polycystiska ovarier” syftar på att äggstockarna innehåller många omogna äggblåsor (folliklar). Många kvinnor med PCOS har nedsatt insulinkänslighet och omkring hälften är överviktiga eller feta. Tidigare studier visar också att kvinnor med PCOS har en ökad benägenhet att lagra fett över magen, vilket är associerat med ökad sjukdomsrisk. Insulinkänsligheten och övervikten gör att dessa kvinnor ofta drabbas av diabetes och på sikt även av hjärt-kärlsjukdomar. Symtomen börjar ofta i samband med puberteten och tilltar om kvinnorna går upp i vikt.

Ett mål med denna avhandling var att utveckla nya råttmodeller som återspeglar de hormonella och metabola störningarna vid PCOS. Eftersom de flesta kvinnor med PCOS börjar utveckla sina symtom under tidig pubertet, i samband med att manligt könshormon börjar frisättas, gav vi honråttor androgener med start före puberteten för att studera om de i vuxen ålder utvecklar ett tillstånd som liknar det hos kvinnor med PCOS. De vuxna honråttorna fick oregelbunden menstruation och äggstocksförändringar liknande de hos kvinnor med PCOS samt metabola rubbningar, såsom insulinkänslighet och fetma med förstörade fettceller.

Orsaken till PCOS är fortfarande oklar. Dessa kvinnor behandlas ofta med olika läkemedel för att lindra syndromets symtom, men behandlingen medför ofta biverkningar. En hög aktivitet i det sympatiska, icke-viljestyrda, nervsystemet tros vara en bidragande faktor till PCOS. Lågfrekvent elektroakupunktur och fysisk träning representerar två icke-farmakologiska behandlingsalternativ som kan påverka både det sympatiska nervsystemet och hormonutsöndringen med få biverkningar. Elektroakupunktur innebär att akupunktur nålarna, som sätts i muskulaturen, stimuleras med svag ström och på så sätt ger effekter som delvis liknar muskelarbete. Vi ville därför utvärdera effekten av elektroakupunktur och fysisk träning i vår råttmodell för PCOS med avseende på både metabola störningar och äggstocksruddningar. PCOS-råttor som sprang i ett hjul fick minskad kroppsvikt, fettmassa och mindre fettceller samt förbättrad insulinkänslighet. PCOS-råttor som fick elektroakupunktur ökade sin känslighet för insulin utan några effekter på kroppsvikt eller kroppssammansättning. I båda behandlingsgrupperna såg vi en förbättring av äggstocksruddningarna.

Ett annat mål med avhandlingen var att i detalj studera fettvävnaden hos kvinnor med PCOS jämfört med kontroller matchade för body mass index (BMI) och ålder. Kvinnorna med PCOS hade en sänkt insulinkänslighet och en störd könshormonbalans jämfört med kontrollgruppen, men trots sofistikerade metoder kunde vi inte visa på någon skillnad i fettfördelning runt buken jämfört med kontroller. Detta tyder på att de metabola rubbningarna associerade till PCOS inte är så starkt kopplade till bukfetma som man tidigare trott. Kvinnor med PCOS hade även större fettceller, lägre nivåer av hormonet adiponectin i blodet, och en tendens till lägre aktivitet av ett enzym (lipoprotein lipas, LPL) som är ansvarigt för leverans av fett till fettvävnaden. Förstorade fettceller tillsammans med låga nivåer av hormonet adiponectin och ett stort midjeomfång var de faktorer som var starkast associerade till den minskade insulinkänsligheten hos kvinnor med PCOS och kan därför vara bakomliggande orsaker till denna störning.

Sammanfattningsvis spelar androgener troligen en stor roll för utvecklingen av PCOS. Råttmodellerna belyser det nära sambandet mellan överskott av androgener och utvecklingen av äggstocks- och metabola störningar typiska för PCOS. Kvinnor med PCOS är insulinokänsliga och har höga nivåer av androgener trots att de inte skiljer sig från ålders- och BMI-matchade kontroller avseende fettfördelning. Av de variabler vi studerade tycks stora fettceller och funktionella avvikelser i fettvävnaden, men inte höga androgennivåer, vara de främsta orsakarna till dessa kvinnors insulinokänslighet. Resultaten från de djurexperimentella studierna visar att elektroakupunktur och fysisk träning representerar två potentiella behandlingsmetoder vid PCOS med gynnsamma effekter på störningar i både äggstockar och metabolism.

LIST OF PUBLICATIONS

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

- I **A New Rat Model Exhibiting Both Ovarian and Metabolic Characteristics of Polycystic Ovary Syndrome**
Mannerås L, Cajander S, Holmäng A, Seleskovic Z, Lystig T, Lönn M, and Stener-Victorin E
Endocrinology 2007; 148(8):3781-91
- II **Low Frequency Electro-Acupuncture and Physical Exercise Improve Metabolic Disturbances and Modulate Gene Expression in Adipose Tissue in Rats with Dihydrotestosterone Induced Polycystic Ovary Syndrome**
Mannerås L, Jonsdottir IH, Holmäng A, Lönn M, and Stener-Victorin E
Endocrinology 2008; 149(7):3559-68
- III **Acupuncture and Exercise Restore Adipose Tissue Expression of Sympathetic Markers and Improve Ovarian Morphology in Rats with Dihydrotestosterone-Induced PCOS**
Mannerås L, Cajander S, Lönn M, and Stener-Victorin E
Am J Physiol Regul Integr Comp Physiol 2009; 296(4):R1124-31
- IV **Adipose tissue characteristics, but not circulating sex steroids, are central factors in the pathogenesis of insulin resistance in women with polycystic ovary syndrome**
Mannerås-Holm L, Leonhardt H, Kullberg J, Jennische E, Odén A, Holm G, Hellström M, Lönn L, Olivecrona G, Stener-Victorin E, and Lönn M
Manuscript

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CONTENTS

ABSTRACT	5
POPULÄRVETENSKAPLIG SAMMANFATTNING	7
LIST OF PUBLICATIONS	9
CONTENTS	10
ABBREVIATIONS	12
INTRODUCTION	14
1 POLYCYSTIC OVARY SYNDROME	14
1.1 <i>Definition and prevalence</i>	14
1.2 <i>Pathophysiology</i>	15
1.3 <i>Ovarian dysfunction</i>	20
2 METABOLIC DISTURBANCES IN PCOS	21
2.1 <i>Insulin resistance</i>	21
2.2 <i>Obesity, fat distribution and adipose tissue function and morphology</i>	22
3 ANDROGENS – A KEY COMPONENT IN THE VICIOUS CIRCLE OF PCOS	31
4 ANIMAL MODELS FOR PCOS	33
5 TREATMENT FOR PCOS	34
5.1 <i>Effects and mechanisms of physical exercise</i>	35
5.2 <i>The hypothetical mode of action of acupuncture</i>	36
AIMS	40
1 OVERALL AIM	40
2 SPECIFIC AIMS	40
METHODOLOGY	41
1 ETHICS (PAPERS I-IV)	41
2 ANIMALS STUDIES (PAPERS I-III)	41
2.1 <i>Animal models</i>	41
2.2 <i>Study design</i>	42
2.3 <i>Treatment (papers II and III)</i>	42
3 HUMAN STUDY (PAPER IV)	43
3.1 <i>Subjects</i>	43
3.2 <i>Samples</i>	44
4 SUMMARY OF THE METHODS (PAPERS I-IV)	44
5 ESTROUS CYCLICITY AND OVARIAN MORPHOLOGY (PAPERS I-III)	45
5.1 <i>Estrous cyclicity (papers I, II and III)</i>	45
5.2 <i>Ovarian morphology (papers I and III)</i>	46
6 ASSESSMENT OF BODY COMPOSITION (PAPERS I-IV)	46
6.1 <i>DEXA (papers I and II)</i>	46

CONTENTS

6.2 MRI (papers I and IV).....	47
7 EUGLYCEMIC-HYPERINSULINEMIC CLAMP (PAPERS I, II AND III)	47
8 ADIPOCYTE SIZE DETERMINATION (PAPERS I, II AND IV)	48
9 REAL TIME RT-PCR (PAPERS II AND III)	49
10 LPL-ACTIVITY IN ADIPOSE TISSUE (PAPER IV)	50
11 IMMUNOHISTOCHEMISTRY – MACROPHAGE DENSITY (PAPER IV).....	50
12 ANALYTICAL METHODS (PAPERS I, II AND IV).....	51
12.1 Immunoassays.....	51
12.2 Mass spectrometry (paper IV).....	52
13 STATISTICAL ANALYSES (PAPERS I-IV).....	52
13.1 Animal Studies (papers I-III)	52
13.2 Human study (paper IV)	53
SUMMARY OF RESULTS.....	54
1 PAPER I.....	54
1.1 Body composition and metabolic features.....	54
1.2 Estrous cyclicity and ovarian morphology.....	54
2 PAPERS II AND III	55
2.1 Body composition and metabolic features.....	55
2.2 Estrous cyclicity and ovarian morphology.....	55
2.3 Gene expression analysis in mesenteric fat.....	55
3 PAPER IV	56
3.1 Paired comparisons.....	56
3.2 Factors associated with insulin sensitivity in women with PCOS	57
DISCUSSION.....	59
THE MAIN FINDINGS OF THE PRESENT THESIS	59
ANDROGENS AND PCOS-RELATED SIGNS AND SYMPTOMS	59
ADIPOSE TISSUE AND INSULIN RESISTANCE IN PCOS	61
EFFECTS OF EA AND EXERCISE ON METABOLIC DISTURBANCES AND OVARIAN DYSFUNCTION	63
CONCLUDING REMARKS	65
FUTURE PERSPECTIVES	66
ACKNOWLEDGMENTS.....	68
REFERENCES	71

ABBREVIATIONS

ACTH	Adrenocorticotrophic hormone
ADRB3	Beta 3 adrenergic receptor
AES	Androgen Excess and PCOS Society
AMH	Anti-müllerian hormone
AR	Androgen receptor
ASRM	American Society for Reproductive Medicine
ATGL	Adipose triglyceride lipase
BMC	Bone mineral content
BMI	Body mass index
CGRP	Calcitonin gene-related peptide
CLS	Crown-like structure
CNS	Central nervous system
CRF	Corticotrophin-releasing factor
CRP	C-reactive protein
CT	Computed tomography
C _T	Cycle threshold
CVD	Cardiovascular disease
DEXA	Dual energy X-ray absorptiometry
DHEA	Dehydroepiandrosterone
DHEAS	Dehydroepiandrosterone sulfate
DHT	Dihydrotestosterone
EA	Electro-acupuncture
EIA	Enzyme immunoassay
ESHRE	European Society for Human Reproduction and Embryology
FFA	Free fatty acids
FSH	Follicle stimulating hormone
GC-MS	Gas chromatography-mass spectrometry
GDR	Glucose disposal rate
GIR	Glucose infusion rate
GnRH	Gonadotropin-releasing hormone
HOMA	Homeostasis model assessment
HPA	Hypothalamic-pituitary-adrenal
HPO	Hypothalamic-pituitary-ovarian
HSL	Hormone-sensitive lipase
IGF-1	Insulin-like growth factor 1

ABBREVIATIONS

IL-6	Interleukin 6
LBM	Lean body mass
LDA	Low-density array
LH	Luteinizing hormone
LPL	Lipoprotein lipase
MCP-1	Monocyte chemoattractant protein-1
MGL	Monoacylglycerol lipase
MIF	Migration inhibitory factor
MIP-1 α	Macrophage inflammatory protein 1 α
mRNA	messenger ribonucleic acid
MSH	Melanocyte-stimulating hormone
MRI	Magnetic resonance imaging
NGF	Nerve growth factor
NIH	National Institutes of Health
NPY	Neuropeptide Y
PCO	Polycystic ovary
PCOS	Polycystic ovary syndrome
POMC	Pro-opiomelanocortin
PPARG/ γ	Peroxisome proliferator-activated receptor gamma/ γ
QUICKI	Quantitative insulin sensitivity check index
RIA	Radioimmunoassay
RT-PCR	Reverse transcriptase polymerase chain reaction
SAA	Serum amyloid A
SD	Standard deviation
SEM	Standard error of the mean
SHBG	Sex hormone binding globulin
T2DM	Type 2 diabetes mellitus
TNF α	Tumor necrosis factor α
UCP2	Uncoupling protein 2
VIP	Vasoactive intestinal peptide
VMC	Vasomotor centre
WHR	Waist-to-hip ratio

INTRODUCTION

1 Polycystic ovary syndrome

Polycystic ovary syndrome (PCOS) is characterized by hyperandrogenism, ovulatory dysfunction and polycystic ovaries (PCO). The hyperandrogenism is caused by excessive ovarian and/or adrenal androgen secretion and is associated clinical manifestations such as hirsutism, acne and male-pattern baldness.¹ Ovulatory dysfunction may include chronic anovulation and is associated with menstrual disturbances and infertility.¹ PCO is characterized by an increased number of small antral follicles with arrested development and a hypertrophied theca cell layer.²

In addition to hirsutism, irregular menses, and infertility, women with PCOS display a number of metabolic abnormalities including hyperinsulinemia, insulin resistance, dyslipidemia, and obesity.³ All these features are components of the metabolic syndrome, and women with PCOS are therefore at risk of developing type 2 diabetes (T2DM) which, in turn, puts them at increased risk of developing cardiovascular disease (CVD).³

1.1 Definition and prevalence

PCOS was first described in 1935 by Stein and Leventhal, who noticed the association between amenorrhea, hirsutism, and enlarged PCO.⁴ However, the definition of the syndrome is still the subject of some debate and its pathogenesis remains unknown.

Three different sets of standard diagnostic criteria have been proposed, reflecting the heterogeneity of the syndrome (Table 1). The first attempt to define PCOS was made during an expert conference held at the National Institutes of Health (NIH) in 1990, and this included both hyperandrogenism and ovulatory dysfunction.⁵ In 2003, the Rotterdam conference, sponsored by the European Society for Human Reproduction and Embryology (ESHRE) and the American Society for Reproductive Medicine (ASRM), broadened the definition of PCOS by including PCO morphology, and the requirement for at least two of the three diagnostic features.⁶ Finally, the Androgen Excess and PCOS Society (AES) proposed new diagnostic criteria in 2006, which made hyperandrogenism fundamental and excluded the phenotype of the non-hyperandrogenic woman with ovulatory dysfunction which is included by the Rotterdam criteria.⁷ All three sets of PCOS diagnostic criteria require the exclusion of other disorders that cause hyperandrogenism and ovulatory dysfunction, e.g. non-

classic congenital adrenal hyperplasia, hyperprolactinemia, Cushing's syndrome, and androgen-secreting tumors.⁵⁻⁷

Table 1. PCOS diagnostic criteria.

Definition	Diagnostic criteria ^A	Phenotypes
NIH 1990	Requires the presence of 1) Hyperandrogenism (HA) ^B and 2) Ovulatory dysfunction (OD) ^C	1. HA + OD
Rotterdam 2003	Requires the presence of at least two of 1) Hyperandrogenism ^B 2) Ovulatory dysfunction ^C 3) PCO morphology ^D	1. HA + OD + PCO 2. HA + OD 3. HA + PCO 4. PCO + OD
AES 2006	Requires the presence of 1) Hyperandrogenism ^B and 2) Ovarian dysfunction (ovulatory dysfunction ^C or PCO morphology ^D)	1. HA + OD + PCO 2. HA + OD 3. HA + PCO

^A Exclusion of other disorders causing hyperandrogenism and ovulatory dysfunction is a criterion of all three definitions

^B Clinical and/or biochemical signs of hyperandrogenism

^C Oligomenorrhea, amenorrhea, oligoovulation, and anovulation

^D Twelve or more 2-9 mm follicles and/or at least one enlarged ovary (>10 ml)

Estimates of the prevalence of PCOS depend on the definition used. According to the NIH criteria, 6-8% of women in the general population have PCOS.⁸ A higher figure is obtained using the broader Rotterdam criteria. A recent Australian study, including primarily Caucasians, found that the prevalence of PCOS under the Rotterdam and AES criteria was almost twice that produced by the NIH criteria.⁹

1.2 Pathophysiology

The pathogenesis of PCOS is thought to be complex and multifactorial but is poorly understood. The heterogeneity of the syndrome may well reflect multiple underlying mechanisms (Figure 1). Androgens and insulin are two key endocrine mediators. There is a strong association between hyperinsulinemia and hyperandrogenism, but the mechanisms behind their relationship and their associations with PCOS are not fully understood.¹⁰ Whether hyperandrogenism results from the hyperinsulinemia of insulin resistance, or vice versa, has been debated since the association was first discovered.

The most popular theories that have been put forward to explain the pathogenesis of PCOS include 1) neuroendocrine defects, 2) impaired ovarian steroidogenesis, 3) impaired adrenal androgen production, 4) insulin resistance with compensatory hyperinsulinemia, 5) increased sympathetic activity, and 6) genetic defects (Figure 1).

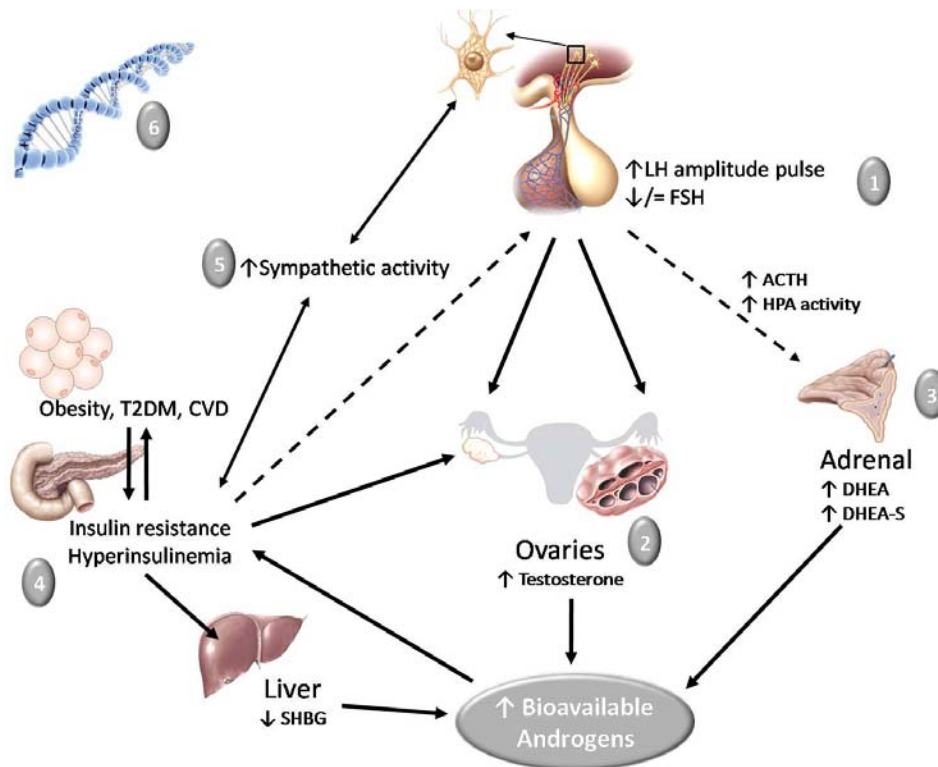


Figure 1. Several theories have been proposed to explain the pathogenesis of PCOS. 1) Neuroendocrine defects, leading to increased pulse frequency and amplitude of LH with relatively low FSH. 2) Intrinsic defects in ovarian androgen production. 3) Alteration in cortisol metabolism and impaired adrenal androgen production. 4) Insulin resistance with compensatory hyperinsulinemia which results in increased ovarian androgen production directly and indirectly via inhibition of hepatic SHBG production. 5) Increased sympathetic nerve activity. 6) Genetic defects. (ACTH – adrenocorticotrophic hormone, CVD – cardiovascular disease, DHEA – dehydroepiandrosterone, DHEAS – dehydroepiandrosterone sulfate, FSH – follicle stimulating hormone, HPA – hypothalamic-pituitary-adrenal, LH – luteinizing hormone, SHBG – sex hormone binding globulin, T2DM – type 2 diabetes mellitus)

1.2.1 Neuroendocrine defects

The frequency and amplitude of hypothalamic gonadotropin-releasing hormone (GnRH) secretion vary throughout the menstrual cycle and regulate pituitary luteinizing hormone (LH) and follicle-stimulating hormone (FSH) synthesis and secretion.¹¹ The increase in GnRH frequency seen in the late follicular phase

stimulates LH synthesis prior to the LH surge, while following ovulation, luteal steroids (progesterone and estradiol) slow GnRH pulses to promote FSH synthesis.¹¹ The differential secretion of FSH and LH is critical for follicular development and subsequent ovulation and regulates ovarian production and secretion of sex steroids (estrogens, androgens and progesterone).

In PCOS, the delicate balance of the hypothalamic-pituitary-ovarian (HPO) axis is disturbed, resulting in abnormal follicle growth and maturation, and subsequent oligo-/anovulation. Women with PCOS display abnormal patterns of gonadotropin secretion, characterized by increased LH pulse frequency and amplitude together with normal or low FSH secretion, resulting in an elevated LH/FSH ratio.¹²⁻¹⁴ The abnormal gonadotropin secretion may be due to enhanced pituitary sensitivity to GnRH stimulation or to increased pulse frequency of GnRH secretion.^{12,15} Aberrations in GnRH pulse frequency and inappropriate gonadotropin secretion may also reflect an insensitivity of the GnRH pulse generator to the negative feedback effects of estrogens and progesterone.^{14,16} Excess LH enhances androgen biosynthesis from ovarian theca cells, while the relative FSH deficiency impairs follicular maturation.

1.2.2 Impaired ovarian steroidogenesis

The ovaries are the main source of the excess androgen seen in PCOS. Ovarian steroids are produced by the theca and granulosa cells working together (Figure 2).¹⁷ LH stimulates theca cells to produce androstenedione from cholesterol. This is then converted to estrogens in the granulosa cells by the action of FSH-dependent aromatase (CYP19). In fact, every molecule of estrogen is derived from a molecule of androgen.

The two principal factors influencing the total amount of androgen secreted by the ovary are the total number of theca cells and their steroidogenic capacity, both of which are disturbed in women with PCOS. Many of the follicles in the PCOS ovary show hypertrophy of the theca interna, resulting in a greater number of steroidogenic cells.¹⁸ Increased androgen synthesis and secretion is a consistent phenotype of ovarian theca cells from women with PCOS.^{19,20} In addition, PCOS ovaries demonstrate hyperactivity of several key enzymes in the biosynthesis of androgens (Figure 2).^{19,21,22} The large quantities of androstenedione secreted by the theca cells are metabolized in peripheral tissues, such as adipose tissue, skin and liver, into testosterone and estrogen. In fact, nearly half of the circulating testosterone in women derives from the peripheral metabolism of androstenedione.

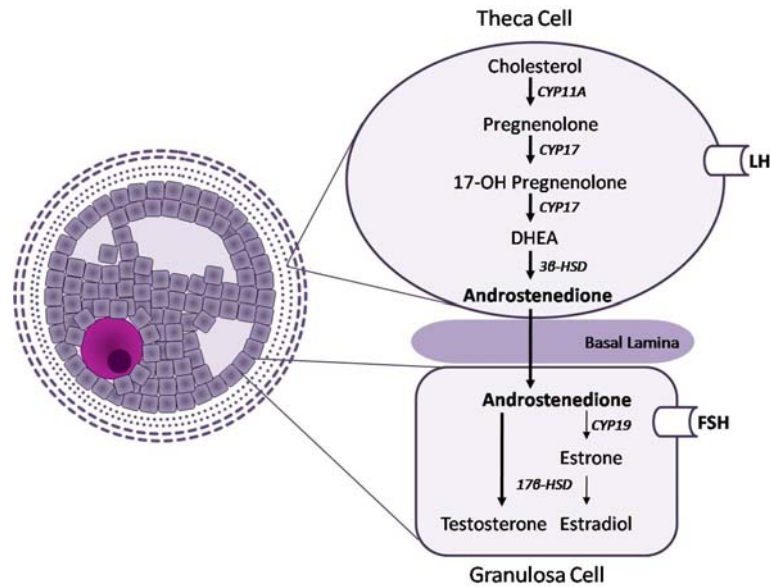


Figure 2. Ovarian steroidogenesis in theca and granulosa cells. LH stimulates theca cells to express the enzymes essential for the production of androstenedione (CYP11A, CYP17, and 3 β -HSD). The androstenedione diffuses across the basal lamina into the granulosa cells where it is metabolized to estradiol in normal ovaries, but in polycystic ovaries (PCO) the androstenedione is metabolized into testosterone to a greater extent. (3 β -HSD – 3 β -hydroxysteroid dehydrogenase, 17 β -HSD – 17 β -hydroxysteroid dehydrogenase, CYP11A – cholesterol side-chain cleavage cytochrome P450, CYP17 – 17 α -hydroxylase/17,20-lyase cytochrome P450, CYP19 – aromatase cytochrome P450, FSH – follicle stimulating hormone, LH – luteinizing hormone)

1.2.3 Impaired adrenal androgen production

The adrenal cortex is the other major site of female androgen production. The adrenal gland utilizes the same steroidogenic pathway as the ovary, but under the endocrine control of adrenocorticotrophic hormone (ACTH) instead of LH (Figure 2). The elevated levels of adrenal androgens such as dehydroepiandrosterone sulfate (DHEAS) seen in women with PCOS suggest that there is an adrenal component to their hyperandrogenism.²³ However, the mechanisms of adrenal hyperandrogenism in PCOS are unclear. Adrenal androgen excess in women with PCOS seems to arise from hypersecretion of adrenocortical products, both basally and in response to ACTH stimulation, rather than from dysfunctions of the hypothalamic-pituitary-adrenal (HPA) axis.^{23,24} It has also been suggested that increased metabolism of cortisol, which leads to decreased negative feedback on ACTH, also contributes to adrenal hyperandrogenism.²⁵

1.2.4 Insulin resistance with compensatory hyperinsulinemia

Insulin plays both direct and indirect roles in the pathogenesis of androgen excess in PCOS. Although women with PCOS have peripheral insulin resistance, ovarian steroidogenesis appears to be hypersensitive to insulin.²⁶ Insulin acts synergistically with LH to enhance theca cell androgen production in women with PCOS by activating a specific signaling pathway via its own receptor.²⁷⁻²⁹ In addition, insulin can stimulate human theca cell proliferation,³⁰ and can also enhance ovarian growth and follicular cyst formation in rats.³¹

Hyperinsulinemia may also have adverse effects in women with PCOS through its action at non-ovarian sites including the liver, adrenal glands and pituitary.^{27,32-35} Insulin has an inhibitory effect on hepatic sex hormone binding globulin (SHBG) production in women with PCOS, increasing the proportion of biologically available androgens and thereby contributing to hyperandrogenism.^{32,33} Insulin also potentiates ACTH-mediated adrenal androgen production.³⁴ The concept that hyperinsulinemia affects GnRH pulse frequency and inappropriate gonadotropin secretion in PCOS by acting at pituitary level is mainly based on *in vitro* studies in which insulin has been shown to increase LH secretion from cultured rat pituitary cells.³⁵ In contrast to animal studies, clinical investigations have not been able to demonstrate that insulin affects gonadotropin secretion in women with PCOS.³⁶⁻³⁸ However, acute administration of insulin in lean, normal young women increases LH pulse frequency, suggesting that there is a functional link between insulin and the activity of the HPO axis.³⁶

Reducing insulin resistance with insulin-sensitizing drugs, such as metformin, produces a moderate improvement in hyperandrogenemia and hyperandrogenemia and, consequently, ovulatory function.³⁹ This suggests that insulin has an important pathophysiological role in PCOS, although its role in neuroendocrine dysfunction remains unclear.

1.2.5 Increased sympathetic activity

Altered activity in the sympathetic nervous system may play a part in the etiology of PCOS.⁴⁰ Hyperandrogenism, insulin resistance with compensatory hyperinsulinemia, and central obesity, are all PCOS-related factors associated with an increased activity in the sympathetic nervous system.⁴¹⁻⁴⁴ The hypothesis that the sympathetic nervous system has a role in the etiology of PCOS is further strengthened by the finding of a greater density of catecholaminergic nerve fibers in PCO^{45,46} and altered catecholamine metabolism in adolescents with PCOS.⁴⁷ It was recently shown that women with PCOS have increased production of ovarian nerve growth factor (NGF).⁴⁸ NGF is a strong marker for sympathetic nerve activity. These results suggest

that overproduction of ovarian NGF is a factor in human PCO morphology. Studies using indirect markers of autonomic function – heart rate variability and heart rate recovery after exercise – have shown that women with PCOS have increased sympathetic and decreased parasympathetic components.⁴⁹⁻⁵¹ We have recently demonstrated, by direct intraneural recordings, that women with PCOS have increased sympathetic activity, which, in turn, was correlated to high testosterone levels.⁵² However, further work is needed to clarify whether increased sympathetic activity is a cause of PCOS or one of its consequences, e.g. via hyperandrogenemia.

1.2.6 Genetic defects

Interaction between multiple genetic and environmental factors is probably necessary for the development of PCOS. Several lines of research suggest that there is a genetic component to the pathophysiology of the syndrome.⁵³⁻⁶⁰ The familial basis of PCOS has been established by multiple family studies that demonstrate clustering of the disorder, with increased prevalence of hyperandrogenism, metabolic disturbances and PCO morphology in female relatives of affected women.^{53,54,57-59} In addition, male relatives of women with PCOS are at greater risk of developing insulin resistance and other metabolic disturbances, suggesting that factors associated with the condition can be passed down to sons as well as to daughters.⁵⁶ Studies of the prevalence of PCOS in twins also suggest that genetic factors contribute to the pathogenesis.⁵⁵ Candidate genes include those involved in the biosynthesis and action of androgens, those related to insulin resistance and those encoding inflammatory cytokines.⁶⁰ Despite a large number of association studies, no single gene has yet been established as a significant factor in the pathogenesis of PCOS, and the mode of inheritance of PCOS remains unclear.

1.3 Ovarian dysfunction

Women with PCOS typically have enlarged ovaries with a hypertrophied stroma and an increased number of small antral follicles predominantly located peripherally under a thickened capsule.^{2,61-63} Ovulatory dysfunction in women with PCOS is due to disturbances in folliculogenesis, the process during which small primordial follicles develop into large preovulatory follicles and which culminates in ovulation.² The early follicular growth is accelerated, but the follicles arrest in their development when they reach 2-9 mm in diameter (small antral follicles), a phase during which the selection of a dominant follicle would normally occur.^{2,62-65} It is likely that the abnormal endocrine environment in women with PCOS, particularly hypersecretion of LH, insulin and androgens, along with relative FSH deficiency, impairs the development of the maturing follicle.^{65,66} Studies in rhesus monkeys have shown that short-term androgen exposure promotes early follicular growth to the stage of pre-antral and small antral

follicles.⁶⁷ Additionally, androgen excess, together with LH and insulin, may also be involved in the inhibition of follicular maturation towards the dominating stage.⁶⁸ In addition, it has been suggested that reduced levels of oocyte-secreted growth factors contribute to the enhanced early folliculogenesis.⁶⁹ An excess of small antral follicles leads to increased production of anti-müllerian hormone (AMH) by granulosa cells which in turn interferes with follicular FSH responsiveness and follicular maturation.⁷⁰ The raised insulin levels commonly observed in women with PCOS may further contribute to follicular arrest as well as to increased androgen production.^{27,71}

2 Metabolic disturbances in PCOS

Women with PCOS often have metabolic disturbances.³ Insulin resistance with compensatory hyperinsulinemia is a central feature of PCOS as well as of the metabolic syndrome. In fact, the prevalence of metabolic syndrome in women with PCOS is significantly greater than that seen in the general population,⁷²⁻⁷⁵ and women with PCOS are at increased risk of developing T2DM.^{76,77} Hyperandrogenemia has also been suggested as an etiological component of the female metabolic syndrome.^{78,79} As women with PCOS have an increased prevalence of several risk factors of CVD, such as T2DM, dyslipidemia, hypertension and obesity, they would be expected to be at increased risk of CVD.³ However, no studies, and in particular no long-term studies, have shown a convincing link between PCOS and CVD.³

2.1 Insulin resistance

Normal glucose homeostasis is a function of the delicate balance between insulin action in the target tissues and insulin secretion by the pancreatic β -cells. The primary target tissues of insulin are skeletal muscle, liver and adipose tissue. Of these, skeletal muscle accounts for 85% of whole body insulin-stimulated glucose uptake,⁸⁰ which might lead one to think that skeletal muscle is the only important target for glucose homeostasis. However, adipose tissue is increasingly being recognized as playing a central role in determining whole body insulin sensitivity.⁸¹

Insulin resistance, defined as an impaired biological response to insulin, along with its compensatory hyperinsulinemia, are hallmarks of PCOS, which puts women with this condition at an increased risk of impaired glucose tolerance and T2DM. In fact, studies have shown that 30-40% of women with PCOS have impaired glucose tolerance, and as many as 10% develop T2DM by the age of 40.^{76,77} This may partly be due to defects in insulin secretion and reduced hepatic insulin clearance.⁸²⁻⁸⁴

Insulin stimulates ovarian androgen production and reduces hepatic SHBG synthesis, thereby increasing total and free bioavailable androgens (Figures 1 and 6).²⁸ The

association between insulin resistance and hyperandrogenism was first noted in 1921 by Archard and Thiers.⁸⁵ The presence of hyperinsulinemia in women with PCOS was first established in 1980.⁸⁶ Subsequent studies have demonstrated insulin resistance in most women with PCOS.⁸⁷⁻⁹² Although insulin resistance in PCOS is, at least partly, independent of the degree of obesity, as lean women with PCOS are also more insulin-resistant than weight-matched controls, obesity seems to increase insulin resistance and hyperinsulinemia.^{87,88,91} In addition, women with central fat distribution are more insulin-resistant than women with generalized/peripheral fat distribution.^{93,94} It has been suggested that women with PCOS have PCOS-specific insulin resistance, or intrinsic insulin resistance, which is aggravated by obesity.

Non-metabolic actions of insulin, such as mitogenesis and steroidogenesis, seem to be normal in PCOS, whereas the effects of insulin on glucose and lipid metabolism are impaired in the known target tissues, resulting in reduced whole-body insulin sensitivity.⁹⁵⁻⁹⁷

The number and the affinity of the insulin receptors seem to be normal in most target tissues studied in women with PCOS.^{96,97} The molecular mechanisms of insulin resistance in PCOS primarily involve post-binding defects in the insulin-receptor signaling pathway in adipocytes and in skeletal muscle.^{96,97} Potential molecular defects in insulin signaling that have been identified include increased serine phosphorylation of the insulin receptor and insulin receptor substrate 1, which inhibits intracellular transmission of the insulin message.^{96,97}

In vitro studies indicate that androgens can directly induce selective insulin resistance in the adipocytes of women by acting via the androgen receptor.⁹⁸ Similar results have been obtained in rat skeletal muscle, i.e. testosterone exposure impairs insulin signaling transduction,⁹⁹ and similar signaling impairment has been observed in skeletal muscle from PCOS patients.¹⁰⁰ Moreover, testosterone exposure in female rats has been shown to reduce whole-body insulin sensitivity by modifying skeletal morphology – reducing the number of insulin-sensitive fibers, increasing the number of less insulin-sensitive muscle fibers, impairing glycogen synthase activity, and reducing capillary density.¹⁰¹⁻¹⁰⁴ However, these morphological alterations have not been observed in women with PCOS.¹⁰⁵

2.2 Obesity, fat distribution and adipose tissue function and morphology

It is well known that obesity has a negative impact on metabolic function,¹⁰⁶ although there are also metabolically obese individuals of normal weight.¹⁰⁷ However, not all types of obesity are harmful.¹⁰⁸ Men and women have strikingly different fat distributions, suggesting that sex steroids influence body composition.¹⁰⁹ Men tend to accumulate abdominal fat (android fat distribution), while women tend to accumulate

fat in the gluteo-femoral region (gynoid fat distribution) (Figure 3). In addition, men have a greater tendency than women to accumulate fat in the visceral depot.^{110,111} However, the android distribution can be found in women and the gynoid distribution can be found in men.¹¹²

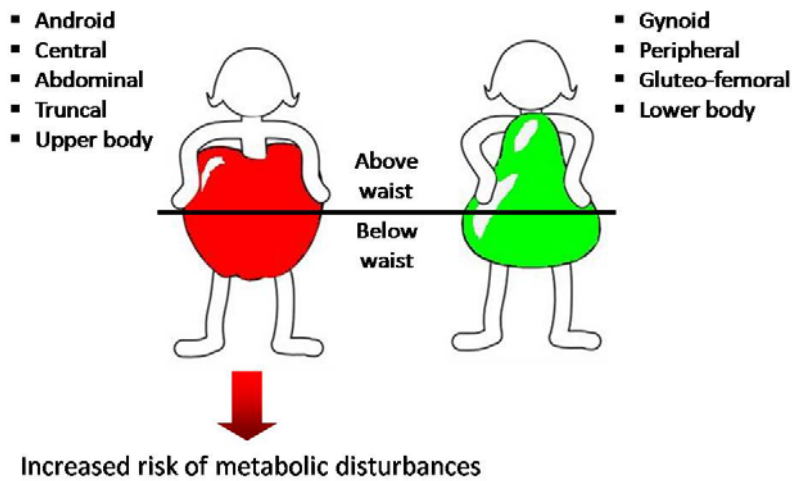


Figure 3. Abdominal, and in particular, visceral fat deposition (above waist, apple) is associated with metabolic disturbances such as insulin resistance, impaired glucose tolerance, dyslipidemia, hypertension, type 2 diabetes mellitus and cardiovascular disease, while accumulation of fat on hips, thighs and buttock (below waist, pear) is less harmful from a metabolic point of view.

The observation that body fat distribution, android versus gynoid, has an impact on metabolic function was described by Vague over half a century ago.¹¹² Since then, several cross-sectional and prospective studies of adipose tissue distribution have been performed.¹¹³ These studies have shown that excess central/visceral as opposed to peripheral deposition of fat is associated with insulin resistance and related metabolic complications such as impaired glucose tolerance, T2DM, dyslipidemia, hypertension and CVD (Figure 3).¹¹⁴⁻¹²¹

Traditional anthropometric measurements, including waist circumference, hip circumference and sagittal diameter, cannot distinguish visceral from subcutaneous abdominal fat.¹²² In contrast, imaging techniques, such as magnetic resonance imaging (MRI) and computed tomography (CT), allow direct assessment of visceral versus subcutaneous fat in the abdominal compartment with high precision and reproducibility.¹²² A number of studies using these techniques have shown that excess visceral fat in particular is associated with a disturbed metabolic profile in both men and women, including women with PCOS.¹²³⁻¹²⁷ However, the association between

visceral fat deposition and insulin resistance has been questioned, while the importance of excess abdominal subcutaneous adipose tissue in this context has been emphasized.¹²⁸

2.2.1 The influence of obesity in PCOS

It is well established that women with PCOS are prone to develop obesity.¹²⁹ The prevalence of overweight and obesity in women with PCOS varies between countries and ethnic groups but may be as high as 75%.¹²⁹ Hyperandrogenemia *per se* may favor enlargement of the visceral fat depot in women.^{130,131} Several investigations report that women with PCOS, regardless of body mass index (BMI), show an android adipose tissue distribution with fat accumulation on the trunk and in visceral depots, possibly in part explaining insulin resistance in these women.^{93,94,132-136} These studies have used different techniques/tools for assessing adiposity and fat distribution, such as simple anthropometric measurements, assessments by lipometer,¹³⁵ ultrasonography,^{133,136} or dual energy X-ray absorptiometry (DEXA),^{93,94,132,134} each with its own advantages and disadvantages. In contrast, a recent study using MRI concluded that women with PCOS and controls matched for BMI and fat mass have similar body fat distribution despite showing significant differences in insulin resistance.¹³⁷ In fact, few detailed controlled studies of body fat distribution and metabolic abnormalities have been carried out in women with PCOS. However, visceral fat mass, assessed by CT, has been reported to be a marker for metabolic disturbances in women with PCOS.¹²⁶

It is possible that the increasing global prevalence of obesity may play a key role in promoting the development of PCOS in susceptible individuals.¹²⁹ In addition, there is no doubt that obesity aggravates preexisting clinical, hormonal and metabolic features in most women with PCOS.¹²⁹ Insulin resistance seems to be a central and independent feature of PCOS, but it is aggravated by obesity, particularly in the abdominal phenotype.^{87,88,91,93,94} Obesity is associated with reduced SHBG levels in both women and men, which may lead to an increased fraction of free androgens.¹³⁸ Unsurprisingly, obese women with PCOS typically have lower plasma SHBG levels and higher free androgen levels than their normal weight counterparts.^{139,140} Furthermore, body fat distribution has been shown to substantially affect SHBG and androgen concentrations. Women with central obesity usually have lower SHBG and higher androgen concentrations than their age- and weight-matched counterparts with peripheral obesity.^{138,141} Women with central obesity produce more testosterone than those with peripheral obesity.¹⁴² Reproductive disturbances, such as menstrual irregularity and anovulatory infertility, are more frequent in obese women than in normal-weight women with PCOS.^{139,143} Obesity, in fact, has a negative impact on reproductive function independently of PCOS.¹⁴⁴ Obese women with PCOS also have

more difficulty conceiving and respond less well to the pharmacological induction of ovulation.¹⁴⁵⁻¹⁴⁸

The impact of obesity in PCOS is further illustrated by the effects of weight loss in obese PCOS patients – reduced circulating androgens and raised SHBG,¹⁴⁹ reduced ovarian volume and follicle count,¹⁵⁰ enhanced insulin sensitivity and reduced hyperinsulinemia,^{149,151,152} and improved menstrual cyclicity and fertility rates.^{149,150} Obesity and possibly adipose tissue related factors may therefore play a pivotal role in the promotion or the maintenance of PCOS. In addition, abdominal fat distribution may aggravate the already adverse endocrine and metabolic profile.¹²⁹

2.2.2 Why is visceral fat accumulation harmful?

Several hypotheses have been proposed as explanations for the deleterious effects of visceral fat on metabolic function. These hypotheses are not mutually exclusive.

Firstly, visceral adipose tissue, located inside the abdominal cavity, is drained by the portal venous system and is therefore the only fat depot that has a direct connection to the liver.¹⁵³ The anti-lipolytic effect of insulin is weaker and the lipolytic effect of catecholamines is stronger in visceral adipocytes than they are in subcutaneous adipocytes, making visceral fat more metabolically active than subcutaneous fat.^{154,155} Therefore, accumulation of visceral fat may increase the delivery of free fatty acids (FFA) via the portal vein to the liver, which may alter hepatic function. An increase in the hepatic production of triglyceride-rich lipoproteins and glucose as well as reduced hepatic insulin clearance have been reported. Acting together, these changes promote dyslipidemia, hyperinsulinemia and glucose intolerance.^{155,156}

Secondly, adipose tissue is an endocrine organ, releasing numerous bioactive substances and pro-inflammatory cytokines. These adipokines have potent effects on adipose tissue and on the metabolism and insulin sensitivity of peripheral tissues. Abdominally obese individuals, with a predominantly visceral fat deposition, have altered plasma levels of adipokines such as interleukin 6 (IL-6),¹²⁷ adiponectin,¹⁵⁷ and tumor necrosis factor α (TNF- α),¹⁵⁸ all of which are implicated in insulin resistance. Therefore, depot-related differences in the production and release of certain adipokines may be involved in the development of obesity-related disease, including insulin resistance.¹⁵⁹⁻¹⁶¹

Thirdly, the deposition of lipids in visceral adipose tissue may be regarded as ectopic fat accumulation. Subcutaneous adipose tissue storage capacity may be reached after a prolonged period of positive energy balance.¹⁶² Consequently, visceral adipose tissue, skeletal muscle, liver and possibly pancreatic β -cells, may accumulate lipids.^{163,164} Ectopic accumulation of fat is associated with insulin resistance.^{156,165-167} In skeletal

muscle, ectopic fat deposition interferes with insulin signaling and glucose uptake.^{163,168}

2.2.3 Adipose tissue as an endocrine organ

The bulk of adipose tissue mass consists of adipocytes. In addition to adipocytes, adipose tissue contains fibroblasts, endothelial cells, sympathetic nerve fibers, immune cells (leukocytes, macrophages), and pre-adipocytes.¹⁶⁹

Adipose tissue is adapted for its main functions – the storage and mobilization of energy, but it also provides thermal and mechanical insulation. Today, adipose tissue is also considered to be an important endocrine organ producing and secreting numerous bioactive peptides and proteins, collectively termed adipokines.¹⁷⁰ Adipokines act at both local (autocrine/paracrine) and systemic (endocrine) levels, allowing crosstalk between adipose tissue and organs.¹⁷⁰ Adipokines enable adipose tissue to play an important role in the regulation of metabolism and inflammation. The key event that established adipose tissue as an endocrine organ was the discovery of leptin in 1994.¹⁷¹ Since then, over 100 adipose tissue-derived factors have been identified as adipokines, all playing a central role in whole body homeostasis by influencing a variety of biological and physiological processes, including food intake, regulation of energy balance, inflammation and acute-phase response, insulin sensitivity, lipid metabolism, angiogenesis, regulation of blood pressure, and coagulation.¹⁶⁹ It should be noted that non-adipose tissues also secrete several adipokines and therefore it is not always easy to determine the specific contribution of adipose tissue to circulating levels of these factors. In addition, there are depot-specific differences in the secretion of adipokines.^{172,173} Furthermore, some adipokines are produced by fat cells within adipose tissue, while others are mainly produced by the stromal-vascular cells.¹⁷² Adiponectin and leptin are thought to be exclusively produced by adipocytes.¹⁷⁴

In addition to their role in increasing insulin resistance and other metabolic abnormalities, some adipokines, when secreted abnormally from adipose tissue, may also influence adrenal and ovarian function.¹⁷⁵ The release of several adipokines has been shown to be disturbed in women with PCOS, but many of these changes seem to be a reflection of the degree of obesity or insulin resistance rather than PCOS *per se*.¹⁷⁵ Leptin is an important regulator of energy homeostasis, but it is also an important hormone in a variety of physiological processes, including gonadal function and reproduction.^{176,177} Although most studies show that leptin levels in women with PCOS are similar to those seen in weight/BMI-matched controls,¹⁷⁸⁻¹⁸⁰ increased leptin levels characteristic of obesity may contribute to the ovulatory dysfunction of PCOS.¹⁷⁵⁻¹⁷⁷ Adiponectin, an adipokine exclusively produced in adipose tissue, has

important insulin-sensitizing, anti-inflammatory and anti-atherosclerotic properties.¹⁸¹ In contrast to many other adipokines, levels of circulating adiponectin are inversely related to body weight, insulin resistance and T2DM.¹⁸² As PCOS is strongly associated with insulin resistance and obesity, decreased circulating adiponectin levels might be expected in women with PCOS. Although there are conflicting results regarding adiponectin levels in women with PCOS, a recent meta-analysis suggests that serum levels of adiponectin are lower in women with PCOS after adjusting for BMI.¹⁸³ Furthermore, both insulin sensitivity and adiposity are strong predictors of levels of circulating adiponectin in PCOS.¹⁸³ In line with this, gene expression of adiponectin is down-regulated in both subcutaneous and visceral fat in women with the syndrome compared to weight-matched controls.¹⁸⁴

2.2.4 Adipose tissue as a steroidogenic organ

Obesity is *per se* a condition of sex hormone imbalance in women, in which increasing body weight increases androgenic status in the presence or absence of PCOS.^{129,185} As well as being viewed as an endocrine organ, adipose tissue can also be regarded as an intracrine organ as regards steroid hormone production, i.e. adipose tissue has the capacity to synthesize and inactivate sex steroids.¹⁸⁶ The activity of various steroidogenic enzymes in adipose tissue is an important determinant of the tissue-specific concentrations of sex steroids and may also influence serum concentrations of these hormones.¹⁸⁷ Local concentrations of androgens in adipose tissue significantly exceed those in the systemic circulation.¹⁸⁸ Although circulating androgens originate mainly from the ovaries and adrenal glands, increased androgen synthesis in adipose tissue could be one mechanism by which obese women with PCOS display increased androgenicity.¹⁸⁷ Adipose tissue also has the ability to produce estrogen via aromatization, and obesity with an increased adipose tissue mass is associated with increased estrogen production.¹⁴² Therefore, increased estrogen production in obese women with PCOS could contribute to gonadotropin imbalance and a consequent increase in ovarian androgen production.

2.2.5 Adipose tissue inflammation and macrophage infiltration

Obesity and T2DM are characterized by a state of chronic low-grade inflammation.¹⁸⁹ This statement is partly based on the observation that obese individuals have increased blood levels of several inflammatory markers such as pro-inflammatory cytokines and acute phase proteins.¹⁸⁹ Central obesity, in particular, has been suggested as a promoter of low-grade chronic inflammation.¹⁹⁰

TNF α was the first adipokine to be suggested as a link between obesity and insulin resistance.¹⁹¹ Since then, adiposity has been linked to increased blood levels of

numerous inflammatory markers including C-reactive protein (CRP), serum amyloid A (SAA) and IL-6.¹⁹²⁻¹⁹⁶ Adipose tissue may contribute substantially to the raised blood levels of several of these markers.¹⁹²⁻¹⁹⁴ Adipose tissue can also have an indirect effect by secreting factors that stimulate the production of inflammatory markers in other organs. For example, IL-6, partly produced and secreted from adipose tissue, is a key inducer of hepatic CRP production.¹⁹⁷ Trayhurn and Wood have proposed that the inflammatory state seen in obesity may essentially be related to local events within adipose tissue and that raised plasma levels of inflammatory cytokines and acute phase proteins result from spill-over from adipose tissue.¹⁷⁰ Hypoxia, which develops when fat mass increases and adipose tissue vascularization deteriorates, could be a key trigger for inflammation-related events.¹⁷⁰

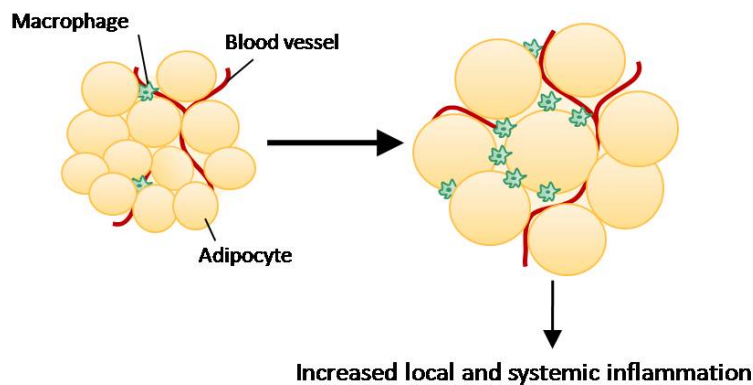


Figure 4. Adipose tissue expansion, especially hypertrophic growth, is associated with an increased infiltration of macrophages.¹⁹⁸ Many of the macrophages are aggregated around adipocytes, forming what are known as crown-like structures (CLS).

It has recently been shown that the adipose tissue of obese animals and humans is infiltrated by macrophages,^{199,200} probably attracted by chemokines secreted by adipose tissue.^{201,202} This suggested mechanism is supported by the fact that obesity is associated with increased levels of the chemokines migration inhibitory factor (MIF),²⁰³ monocyte chemoattractant protein-1 (MCP-1),²⁰⁴ and macrophage inflammatory protein (MIP)-1 α .²⁰⁵ There is increasing evidence that adipose tissue macrophages may be a major source of cytokines and chemokines that further promote a local inflammatory response, resulting in systemic insulin resistance.^{172,201,206,207} Many of the macrophages are aggregated around dead adipocytes, forming so-called crown-like structures (CLS) (Figure 4).¹⁹⁸ Macrophage infiltration and increased levels of chemokines are associated with obesity, fat cell size and insulin resistance.^{199,200,202-204,207,208} However, the hypothesis that greater

abundance of macrophages in adipose tissue contributes to insulin resistance and low-grade inflammation is primarily based on studies on rodents and morbidly obese humans.²⁰⁹

It has also been suggested that PCOS is a proinflammatory condition. Blood levels of inflammatory markers, such as TNF- α , IL-6 and CRP, are higher in women with PCOS than in controls matched for BMI and age,^{94,136,210-219} although the results are not entirely consistent. Most of these studies have reported a close relationship between levels of the inflammatory markers and insulin resistance/obesity, particularly central obesity. Others have suggested that the low-grade chronic inflammation seen in women with PCOS is a function of obesity rather than a consequence of PCOS *per se*.^{94,220,221} Moreover, blood levels of chemokines have been reported to be higher in women with PCOS and hirsutism than in BMI-matched controls^{205,222,223} As with other inflammatory markers, chemokine levels were found to correlate with BMI and central fat mass.^{205,222} It is not known if the increased chemokine levels seen in women with PCOS are the result of an accumulation of macrophages in adipose tissue.

2.2.6 Lipoprotein lipase activity and lipolysis

Fat cells can increase their diameter by a factor of 20 and their volume several thousand-fold. Adipocyte size is mainly determined by lipid droplet size because 95% of the adipocyte consists of triglyceride.²²⁴ Hence, adipocyte size is governed by the balance between storage and mobilization (lipolysis) of triglycerides within the cell. These processes are regulated by lipases involved in the hydrolysis of triglycerides to fatty acids and glycerol. Lipoprotein lipase (LPL) is the enzyme that controls the delivery of fatty acids from circulating triglyceride-rich lipoproteins to the tissue.²²⁵ Mobilization of triglyceride stores within fat cells is catalyzed by three major lipases; hormone-sensitive lipase (HSL), adipose triglyceride lipase (ATGL), and monoacylglycerol lipase (MGL).²²⁶

Previous studies have shown that there are site-specific disturbances in lipolysis in young non-obese women with PCOS. These studies have reported a marked decrease in catecholamine-induced lipolysis in subcutaneous abdominal adipocytes,²²⁷⁻²²⁹ and an increased lipolytic activity in visceral adipocytes, favoring the release of fatty acids from the visceral depot.⁹⁵ Subcutaneous fat cells were enlarged by about 25% in these women, possibly as a result of the lipolytic catecholamine resistance of this depot.²²⁹ No difference in adipocyte size has been observed in other comparisons between small groups of patients with PCOS and controls.^{227,230} Two studies of LPL activity in adipose tissue have produced differing results, one showing reduced, and the other showing similar activity in women with PCOS *vs.* controls.^{231,232}

2.2.7 Adipocyte size

In addition to differences in body fat distribution (android *vs.* gynoid), obesity can be classified according to adipose tissue cellularity. An increase in adipose tissue mass can occur by an increase in the number of adipocytes (hyperplastic growth), an increase in the size of adipocytes (hypertrophic growth) or both. The number of fat cells seems to be fixed during childhood and adolescence and remains fairly constant during adult life, as the rate of formation of new fat cells is counterbalanced by an equal rate of cell death in existing fat cells.²³³ Obesity in adults, therefore, is mainly due to hypertrophic growth rather than hyperplastic growth. Adipocyte number also remains constant after major weight loss, and the reduced fat mass is the result of decreased adipocyte size.²³³

Numerous cross-sectional studies have shown that hypertrophic adipose tissue is associated with metabolic abnormalities such as insulin resistance, T2DM, hepatic lipid accumulation, dyslipidemia, and hypertension (Figure 5).²³⁴⁻²⁴⁰ Moreover, prospective studies have shown that enlargement of subcutaneous abdominal adipocytes is an independent predictor of T2DM.^{241,242}

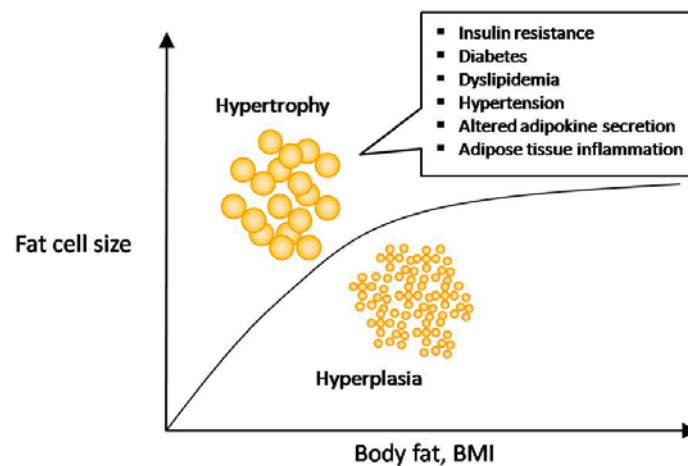


Figure 5. Adipose tissue expands by increasing the size of preexisting adipocytes (hypertrophy), by generating new small adipocytes (hyperplasia), or both. There is a large interindividual variation in mean fat cell size both in lean and obese subjects. Adipose tissue hypertrophy is associated with metabolic abnormalities such as insulin resistance, T2DM, dyslipidemia as well as adipose tissue dysfunction

Cell size is a major determinant of adipocyte and adipose tissue function. Enlargement of adipocytes may reflect failure of the adipose tissue mass to expand further, as well as reflecting an impaired ability to recruit new adipocytes.²⁴³⁻²⁴⁵ This can cause lipid overflow which leads to ectopic fat accumulation and further increase in insulin

resistance. This hypothesis is supported by the observed effects of glitazone [peroxisome proliferator active receptor γ (PPAR γ) agonist, also known as thiazolidinediones (TZD)] treatment, which improves insulin resistance and also seems to increase recruitment of small, new adipocytes.^{246,247} Large adipocytes are also resistant to the antilipolytic effect of insulin,^{248,249} suggesting that adipocyte hypertrophy leads to increased release of FFA.

Increased fat cell size is also associated with an altered expression of adipokines, favoring production and release of proinflammatory mediators acting in an autocrine, paracrine and endocrine manner (Figure 5).^{239,250-253} Consequently, the altered adipokine profile contributes to an inflammatory state in adipose and other tissues, promoting insulin resistance. Moreover, it has been suggested that adipocyte hypertrophy *per se* promotes adipocyte death and macrophage aggregation (Figure 5).^{198,199,250,254} Interestingly, weight loss accompanied by reduced adipocyte size results in reduced macrophage infiltration²⁰² and a beneficial alteration in adipokine secretion.²⁵⁵⁻²⁵⁷

2.2.8 Impaired adipose tissue function in PCOS

Adipose tissue in women with PCOS has been reported to be abnormal in several regards, although these findings are often inconsistent. Low serum adiponectin levels indicate that release of certain adipokines from adipose tissue is disturbed in women with PCOS.¹⁸³ Elevated blood levels of a number of inflammatory mediators, of which several are produced in adipose tissue, have given rise to the hypothesis that low-grade inflammation is present in PCOS.²⁵⁸ Women with PCOS have been found to have BMI-independent enlargement of abdominal subcutaneous adipocytes,^{229,259} presumably because of a low lipolytic activity in this depot.²²⁹ Moreover, increased lipolytic activity has been observed in visceral adipocytes which may promote the release of FFA into the portal circulation.⁹⁵ Reduced adipose tissue LPL activity has been reported in women with PCOS compared to controls.²³² In addition, although skeletal muscle accounts for 85% of whole body insulin-stimulated glucose uptake,⁸⁰ modest changes in adipose tissue glucose turnover can have substantial secondary effects on whole-body glucose metabolism.⁸¹ It is therefore likely that the molecular defects observed in the insulin signaling pathway of adipocytes from women with PCOS may have a significant effect on whole-body insulin resistance.^{96,97}

3 Androgens – a key component in the vicious circle of PCOS

The most consistent biochemical abnormality in PCOS is hypersecretion of androgens, predominantly of ovarian origin. Intra-ovarian hyperandrogenemia in women with PCOS promotes excessive early follicular growth that does not progress

to the dominant stage, leading to anovulation.² At the neuroendocrine level, elevated androgens diminish the sensitivity of the GnRH pulse generator to inhibition by ovarian steroids. This contributes to abnormal secretion of GnRH and gonadotropin, resulting in increased ovarian androgen production and impaired follicle development.^{14,16,260} Testosterone has been found to be the strongest independent factor explaining the high sympathetic nerve activity seen in women with PCOS.⁵²

Abdominal obesity in women is associated with elevated androgen levels and reduced SHBG levels.^{138,142} This, together with the observation that exogenous testosterone treatment in women increases the amount of visceral fat,²⁶¹ indicates that androgens may be an important triggering factor for the abdominal obesity that is often seen in women with PCOS (Figure 6). The detrimental effect of androgens on metabolic function is further illustrated by the observation that hyperandrogenic and anovulatory women with PCOS generally have a more detrimental metabolic profile than non-hyperandrogenic and ovulatory women with PCOS.²⁶² Abdominal obesity, in turn, promotes hyperandrogenemia by an altered release of adipokines which may influence ovarian and adrenal function directly, and also indirectly by the induction of insulin resistance and hyperinsulinemia (Figure 6).^{175,263}

Androgens bind to the androgen receptor which mediates its effects by the transcriptional activation of downstream genes. Androgens may promote insulin resistance by stimulating lipolysis via androgen receptors that are present in greater numbers in visceral adipose tissue than they are in subcutaneous adipose tissue.^{264,265} Consequently androgens promote increased FFA release into the bloodstream.⁹⁵ In addition, androgens may modify skeletal muscle morphology by increasing the numbers of less insulin-sensitive type II muscle fibers, reducing the numbers of insulin-sensitive type I muscle fibers, reducing capillary density and inhibiting glycogen synthase activity.^{101,102} These changes may lead to impaired insulin sensitivity in skeletal muscle (Figure 6). Moreover, androgens have been shown to act directly upon the insulin signaling pathways via their own receptors in both adipocytes and skeletal muscle, inducing insulin resistance (Figure 6).^{98,99} Evidence that excessive androgens in women lead to insulin resistance comes from studies in which women (female-to-male transsexuals, pre- and postmenopausal women) have been treated with exogenous androgens.²⁶⁶⁻²⁶⁹ Moreover, the theory that there exists a close relationship between endogenous androgen excess and the development of insulin resistance is further strengthened by the observation that anti-androgen treatment in hyperandrogenic women can partly reverse insulin resistance.²⁷⁰⁻²⁷² Animal studies in which female rats, sheep and rhesus monkeys have been exposed to androgen excess during intrauterine, neonatal prepubertal or adult life, support the concept that

androgens can induce insulin resistance and obesity, particularly abdominal obesity.^{102,273-277}

Androgen excess of ovarian and/or adrenal origin starting early in life, or even prenatally, initiates a vicious cycle in which hyperandrogenaemia leads to neuroendocrine abnormalities, ovarian dysfunction, abdominal obesity and insulin resistance which in turn further stimulates androgen production (Figure 6).²⁶³

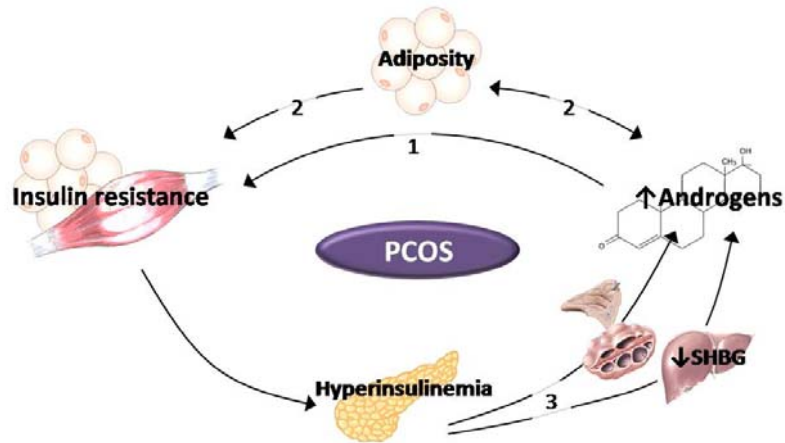


Figure 6. The hallmarks of PCOS – hyperinsulinemia and hyperandrogenism – are part of a vicious circle. 1) Androgens can directly influence insulin action in adipocytes and skeletal muscle. 2) Androgens can also influence insulin sensitivity indirectly via effects on lipid metabolism, adiposity/body fat distribution and adipokine secretion. Adipokines, in turn, may influence ovarian and adrenal function directly. 3) Hyperinsulinemia, compensatory to insulin resistance, enhances hyperandrogenism via direct actions on androgen production and indirectly via an inhibition of hepatic SHBG production

4 Animal models for PCOS

Validated animal models can be used in therapeutic screens, in preclinical trials, and to study the etiology and pathogenesis of complex disorders in ways that are not possible in human studies. In recent decades, a variety of mammalian species, ranging from rodents to non-human primates, have been used to increase our understanding of human PCOS. However, the unclear etiology and pathophysiological mechanisms, as well as the inability to agree on a clinical definition of PCOS, make the development of a single, widely accepted animal model for the syndrome very difficult.

Prenatally androgenized rhesus monkeys and sheep are, so far, the most informative animal models for PCOS, exhibiting both reproductive and metabolic disturbances that mimic those found in women with PCOS.²⁷⁷ However, we do not know how

reliable these animal models are with reference to the origin of human PCOS. Maternal androgens are rapidly converted to estrogens by the aromatase activity of the human placenta which therefore protects the fetus from exposure to excess androgens.²⁷⁸ In addition, the androgen concentrations achieved experimentally in these models are much higher than those observed in pregnant women with PCOS.²⁷⁹ Furthermore, the use of these animals in the study of PCOS is expensive and time consuming due to their long reproductive cycle.

In contrast, the short life cycle of rodents makes them more suitable for studies of both reproductive and long-term metabolic status in a controlled environment. In rats, PCOS-like features may be induced by androgen, estrogen, letrozole, and antiprogestosterone agents, as well as by constant light exposure, during prenatal, neonatal, prepubertal, and adult life.^{273,276,280-285} These exposures all cause disorders of the HPO-axis, but metabolic disturbances are the ones that are commonly investigated. In addition, rodents are multiovulatory with a rapid cycle of 4 to 5 days. Such species differences must be taken into consideration when translating experimental findings to human conditions.

Several existing animal models for PCOS do not meet the full diagnostic criteria, whilst others also include traits that would exclude a diagnosis of PCOS.²⁸⁶

5 Treatment for PCOS

Many women with PCOS require prolonged treatment. The lack of an established pathogenesis has inevitably led to an emphasis on symptomatic treatment. The spectrum of therapeutic options is broad and ranges from lifestyle intervention to specific pharmacological agents. Two of the primary goals of treatment are the normalization of androgen levels and the restoration of reproductive function. However, as the metabolic disturbances of PCOS aggravate many of its typical symptoms, these have become an important target for therapy. First-line treatment in overweight and obese women with PCOS is the reduction of body weight by lifestyle modifications.²⁸⁷ A modest weight loss has been shown to improve hyperandrogenism, ovulatory function as well as the metabolic profile.^{149,151,288} First line pharmacological therapy in PCOS is often combined oral contraception, which improves hirsutism, acne and menstrual cyclicity but may adversely affect insulin sensitivity, lipid profile, coagulation and fertility, especially in obese patients.²⁸⁹ Anti-androgen medication can be used in combination with oral contraceptives to improve clinical signs of hyperandrogenism, but it is not suitable for those trying to conceive. The first-line treatment to induce ovulation in women who wish to become pregnant is clomiphene citrate, which inhibits the action of estrogen on the hypothalamus,

enhancing the secretion of FSH and stimulating the development of ovarian follicles. If clomiphene citrate proves ineffective, further treatment often includes exogenous gonadotropins. Insulin sensitizers, such as metformin, are used to reduce insulin resistance and insulin secretion, and may also resulting in a reduction of ovarian androgen production and a consequent improvement in menstrual cyclicity.³⁹ Although the evidence that metformin is effective is inconclusive, and despite the fact that it is not currently licensed for the management of PCOS, it is widely prescribed.³⁹

Pharmacological approaches are helpful but have adverse effects. Therefore, detailed evaluation of new non-pharmacological treatment strategies, such as acupuncture and physical exercise, is needed. Both repeated acupuncture and physical exercise, in women with PCOS and in women with undefined ovulatory dysfunction, have been shown to provide long-lasting improvement in certain endocrine parameters, as well as on reproductive, metabolic and cardiopulmonary function, with no significant side effects.^{151,288,290-295}

5.1 Effects and mechanisms of physical exercise

Numerous studies have shown that overweight and obesity have detrimental effects on reproductive function. There are few studies on the effects of physical exercise alone in women with PCOS, and most of them are relatively small.²⁹⁶ Most studies have used a combination of lifestyle interventions, including calorie restriction/dietary modification and exercise.²⁹⁶ Lifestyle interventions that include physical exercise improve ovulatory function, androgen status, inflammatory pattern and insulin sensitivity in women with PCOS.^{151,288,293,295,297,298} Weight loss seems to be the main mechanism by which exercise and/or diet improves insulin sensitivity in obese women with PCOS, with subsequent improvements in hormone status and reproductive function.^{151,288,293,295-298} Nevertheless, exercise without weight loss or with only a moderate weight loss can still lead to a significant reduction in visceral fat and an improvement in insulin sensitivity.^{227,299,300}

It has been suggested that altered activity in the sympathetic nervous system plays a part in the pathogenesis of PCOS.⁴⁰ We have recently demonstrated, by direct intraneural recordings, that women with PCOS have increased sympathetic nerve activity,⁵² which can be lowered by both physical exercise and electro-acupuncture (EA), see below.³⁰¹ This is significant, as raised sympathetic activity may contribute to metabolic abnormalities such as insulin resistance.^{302,303}

The mechanisms by which exercise improves insulin sensitivity independently of loss of weight and visceral fat include alterations in muscle cell metabolism and morphology. Exercise can affect blood glucose levels via three main mechanisms – acute stimulation of glucose transport, acute enhancement of insulin action, and long-

term upregulation of the insulin signaling pathway. Myocyte contraction increases glucose uptake by inducing recruitment of a unique GLUT4 intracellular pool to the plasma membrane by a mechanism separate to the insulin signaling pathway.³⁰⁴⁻³⁰⁷ Regular exercise induces changes in the insulin signaling pathway, which acute exercise does not.^{308,309} Exercise also promotes increased muscle blood flow and skeletal muscle capillarization, facilitating the action of insulin and glucose transport into the muscles.^{310,311}

5.2 The hypothetical mode of action of acupuncture

Acupuncture, derived from the Latin words *acus* (needle) and *pungere* (to puncture), is defined as the insertion of needles into the body at specific points (acupoints). After insertion, needles may be further stimulated by manual manipulation or by electrical stimulation (EA). In EA, an electrical current is passed between two needles. It has been suggested that low-frequency (1-15 Hz) EA with repetitive muscle contraction results in activation of physiological processes similar to those resulting from physical exercise.

Acupuncture is a form of sensory stimulation that activates nervous pathways at different levels – peripheral (local), segmental (spinal cord) and central nervous system (CNS) (supraspinal) (Figure 7).

At peripheral level, manual and electrical stimulation of acupuncture needles located in muscle tissue induces the release of a number of neuropeptides from the peripheral nerve terminals into the surrounding area. These include substance P, calcitonin gene-related peptide (CGRP), vasoactive intestinal peptide (VIP) and neuropeptide Y (NPY),³¹²⁻³¹⁴ which increase microcirculation and glucose uptake,^{312,314,315} the latter probably via a reflex response from muscle twitches during manual or electrical stimulation.³⁰⁴ Low-frequency EA activates high- and low-threshold muscle mechanoreceptors that are physiologically activated during muscle contractions.^{316,317} Special attention has been paid to a group of mechanoreceptors in skeletal muscle known as ergoreceptors. These are innervated by thin myelinated A δ afferents and possibly unmyelinated C afferents.^{318,319} The activation of A δ and C fibers by stimulation with acupuncture needles inserted into muscle transmits signals to the spinal cord (segmental level), where the organ in the same area of innervation may be modulated via sympathetic reflexes (Figure 7).³²⁰ After transmission at spinal level, the afferent signals continue to the CNS via ascending pathways. These signals influence various structures in the brain, including the hypothalamus and pituitary, and modulate activity in the nervous and endocrine systems. Significantly, most spinal reflexes are under central control, and both segmental and central mechanisms are probably involved in the overall effect of acupuncture treatment (Figure 7).³²¹

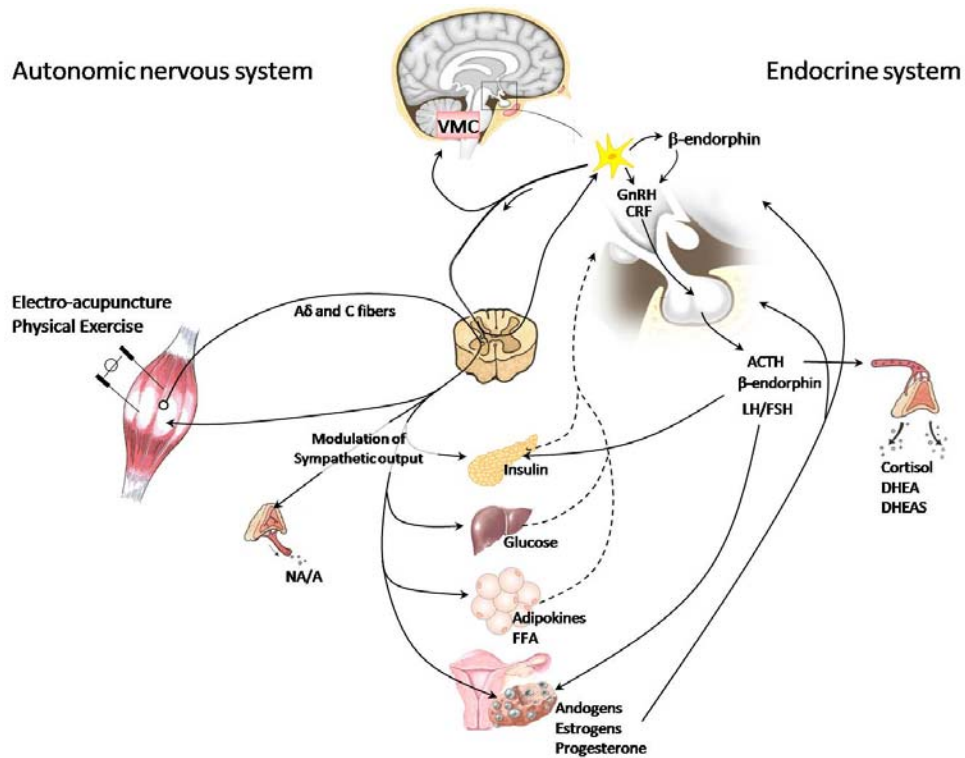


Figure 7. A hypothetical model of the effects of low-frequency EA and physical exercise in PCOS. Muscle contractions caused by low frequency EA and physical exercise excite ergoreceptors in the muscle which in turn activate afferent A δ and C fibers. The signals are transmitted to the spinal cord (segmental level), where the organ in the same area of innervation may be modulated via sympathetic reflexes. After transmission at spinal level the signals continue to the CNS via ascending pathways, where activity in the nervous and endocrine systems may be modulated. The central effects of β -endorphin, produced by hypothalamic neurons, are exerted by the modulation of the autonomic system e.g. via the VMC. β -endorphin may alter the release of CRF and GnRH and may therefore modulate the HPA and HPO axes. CRF promotes the release of β -endorphin and ACTH, which enter the bloodstream and which could have peripheral effects on pancreatic insulin secretion and on the secretion of cortisol and adrenal androgens. GnRH modulates the release of LH and FSH and consequently ovarian sex steroid production. (A – adrenalin, ACTH – adrenocorticotrophic hormone, CNS – central nervous system, CRF – corticotrophin-releasing factor, DHEA – dehydroepiandrosterone, DHEAS – dehydroepiandrosterone sulfate, FFA – free fatty acids, FSH – follicle stimulating hormone, GnRH – gonadotropin-releasing hormone, HPA – hypothalamic-pituitary-adrenal, HPO – hypothalamic-pituitary-ovarian, LH – luteinizing hormone, NA – noradrenalin, VMC – vasomotor centre)

In the CNS, both acupuncture and exercise modulate the production and release of endogenous opioids.^{318,322} β -endorphin, a cleavage product of pro-opiomelanocortin (POMC) which binds to the μ -receptor with high affinity, has been of particular

interest. β -endorphin is produced by hypothalamic neurons and by the anterior pituitary, inducing central and peripheral effects respectively (Figure 7).³¹⁸ These systems work independently, but both can be stimulated by afferent nerve activity.³²³ Both exercise and acupuncture have been shown to modulate β -endorphin levels centrally and peripherally.³²⁴⁻³²⁸

The β -endorphinergic system probably plays a role in a variety of central functions, including reproduction and autonomic function.^{318,329} β -endorphin is produced and released from neurons in the arcuate nucleus of the hypothalamus, which connect to a number of sites in the brain, including all parts of the hypothalamus.³³⁰ Hypothalamic β -endorphin interacts with the HPO axis, by exerting a tonic inhibitory effect on the GnRH pulse generator and pituitary LH release.^{331,332} β -endorphin may also interact with the HPA axis by modulating the release of corticotrophin-releasing factor (CRF). In addition, β -endorphin is a key mediator of change in autonomic function, e.g. by influencing the vasomotor centre (VMC), which leads to a general decrease of sympathetic tone as seen in the regulation of blood pressure, and also by reducing the activity in the sympathetic nervous system (Figure 7).

β -endorphin is also co-released into the bloodstream with ACTH and melanocyte-stimulating hormone (MSH) from the anterior pituitary under the regulation of CRF (Figure 7).³³³ These hormones are transported by the bloodstream to exert their effects on remote target organs. The release of β -endorphin into the bloodstream could theoretically have an effect on the pancreas and could affect the release of insulin via activation of μ -receptors located in the pancreatic β -cells.^{334,335} The release of ACTH affects the HPA axis, altering the secretion of cortisol and adrenal androgens from the adrenal cortex, which then, in turn, affect the negative feed-back system.

5.2.1 PCOS and the potential effects of acupuncture

Disorders of opioid system regulation may explain several characteristics of PCOS, including abnormal gonadotropin secretion, insulin resistance and obesity.³²⁹ Women with PCOS have elevated levels of circulating β -endorphin, and this seems to be independent of BMI, although obesity itself has been associated with increased β -endorphin levels.^{336,337} It is not known whether β -endorphin levels in the CNS are increased.

The role of β -endorphin as an inhibitory modulator of the GnRH pulse generator and pituitary LH release, suggests that PCOS may partly be the result of inadequate central β -endorphin inhibition of GnRH. Evidence that β -endorphin does play a role in the pathogenesis of PCOS and in the dysregulation of GnRH/LH secretion comes from studies in which naltrexone, a μ -receptor antagonist, improved menstrual cyclicity,

induced ovulation and decreased LH levels, the LH/FSH ratio, and testosterone levels.³³⁸⁻³⁴⁰ As the effects of acupuncture may, at least in part, be mediated by modulation of β -endorphin production and secretion, which in turn affects GnRH/LH secretion, it is possible that acupuncture can improve ovulatory dysfunction and decrease ovarian androgen production in PCOS. Acupuncture treatment in women with clearly defined PCOS and undefined ovulatory dysfunction has been shown to have long-lasting effects on endocrine variables, such as a lower LH/FSH ratio, decreased testosterone and β -endorphin concentrations, as well as on anovulation, with no adverse effects.²⁹⁰⁻²⁹² In addition, for the first time we have demonstrated that low-frequency EA *and* physical exercise lowers high sympathetic nerve activity in women with PCOS. Interestingly, in the acupuncture group, decrease in sympathetic nerve activity was associated with decrease in circulating testosterone.³⁰¹ EA could therefore be considered as an alternative to the pharmacological induction of ovulation, but randomized controlled trials are needed.

Opioids can stimulate the production of insulin by the pancreas³³⁴ and inhibit the clearance of insulin by the liver,³⁴¹ thereby contributing to the hyperinsulinemia often seen in PCOS. Several studies have shown that the inhibition of opioid tone results in a reduction of hyperinsulinemia in women with PCOS,³³⁹⁻³⁴¹ probably due to an increased rate of insulin clearance or to increased sensitivity to insulin in target tissues.^{340,341} Interestingly, low-frequency EA has been shown to decrease high circulating β -endorphin concentrations in women with PCOS and may hypothetically decrease hyperinsulinemia and increase insulin clearance and/or increase insulin sensitivity.^{290,292}

In addition, β -endorphin seems to have role in obesity, as plasma levels of β -endorphin are increased in obese subjects.³⁴² Treatment with naltrexone has been shown to have beneficial effects on BMI in women with PCOS.³³⁹ Several studies in humans³⁴³⁻³⁴⁷ and animals³⁴⁸ have shown that EA improves obesity and obesity related-parameters.

In Western medical acupuncture, PCOS is treated by inserting acupuncture needles into the abdominal muscles and into the muscles below the knee in somatic segments linked to ovarian innervation (Th12-L2, S2-S4). Physical exercise combined with acupuncture may hypothetically relieve several PCOS-related symptoms either directly by modulation of sympathetic and endocrine outflow or indirectly by modulation of opioid secretion and release.

AIMS

1 Overall aim

To evaluate the role of androgens and factors related to adipose tissue in the pathogenesis of PCOS and related insulin resistance, and to study the therapeutic impact of low-frequency electro-acupuncture (EA) and physical exercise.

2 Specific aims

- To investigate if continuous administration of dihydrotestosterone (DHT) or letrozole to induce a hyperandrogenic state in female rats from before puberty to adult age, induce disturbances similar to those in human PCOS, including polycystic ovaries, hormonal and metabolic abnormalities, body composition alterations and enlarged adipocytes. (*Paper I*)
- To investigate whether repeated low-frequency (2 Hz) EA and voluntary physical exercise improve metabolic disturbances and ovarian morphology in female rats with PCOS induced by continuous administration of DHT. — The effects of EA and physical exercise on mRNA expression of genes related to insulin resistance, obesity, inflammation, as well as markers of sympathetic nerve activity and the androgen receptor were investigated in the visceral adipose tissue of these rats. (*Papers II and III*)
- To characterize the adipose tissue of women with PCOS compared to BMI- and age-matched controls in terms of distribution, adipocyte size, lipid metabolism, circulating adipokines and macrophage density, and to identify factors, including these characteristics and serum sex steroids, that are associated with insulin sensitivity in women with PCOS. (*Paper IV*)

METHODOLOGY

1 Ethics (papers I-IV)

Experimental studies were approved by the Animal Ethics Committee of the University of Gothenburg. Animals were cared for in accordance with the principles of the Guide to the Care and Use of Experimental Animals (www.sjv.se).

All participants in the human study gave oral and written consent prior to inclusion. The study was conducted in accordance with the Declaration of Helsinki and approved by the Ethics Committee of the University of Gothenburg.

2 Animals studies (papers I-III)

2.1 *Animal models*

In the experimental studies we aimed to induce a hyperandrogenic state from prepubertal age. The initial manifestations of PCOS, such as hirsutism, acne and menstrual disturbances, are frequently peripubertal in onset, suggesting that PCOS has a pre- or peripubertal origin.^{349,350} In addition, the earliest signs of PCOS, including metabolic abnormalities and elevated androgens, can be seen in girls with premature pubarche.^{349,351} Experimentally-induced peripubertal hyperinsulinemia and hyperandrogenism in female rats cause PCO-like ovarian dysfunction, hormonal changes and glucose intolerance.^{285,352} Prepubertal age can therefore be considered to be a sensitive period, and an insult during this period can have pronounced and lifelong effects on both ovarian and metabolic function.

We used letrozole and DHT to induce a state of androgen excess from prepubertal age. Letrozole is a nonsteroidal aromatase inhibitor that blocks the conversion of testosterone to estradiol. The dose of letrozole was based on the study by Kafali et al.²⁸² They have previously shown that oral administration of letrozole produces an ovarian phenotype remarkably similar to that of PCOS, including LH hypersecretion and high testosterone levels in adult female rats.²⁸² However, serum levels of estradiol were greatly diminished, a steroid abnormality not found in PCOS, and metabolic abnormalities were not investigated.²⁸² DHT is a non-aromatizable androgen with a high affinity for the androgen receptor. The chosen dose of DHT was designed to induce a hyperandrogenic state mimicking that seen in women with PCOS, whose plasma DHT levels are approximately 1.7 times higher than those of healthy controls.^{353,354}

2.2 Study design

Female pups were raised with a lactating dam until 21 days of age and were then housed four to five per cage in a controlled environment. Standard principles of laboratory animal care were followed. All experimental procedures were approved by the animal ethics committee of the University of Gothenburg. An overview of the study designs of papers I and II are shown in figures 8A and 8B. A 90-day continuous-release pellet of letrozole (paper I) or DHT (papers I-III) was implanted subcutaneously in the neck at 21 days of age. Controls received identical pellets lacking the bioactive molecule (papers I-III). In papers II and III the rats were implanted with an additional 60-day continuous-release pellet to compensate for weight gain.

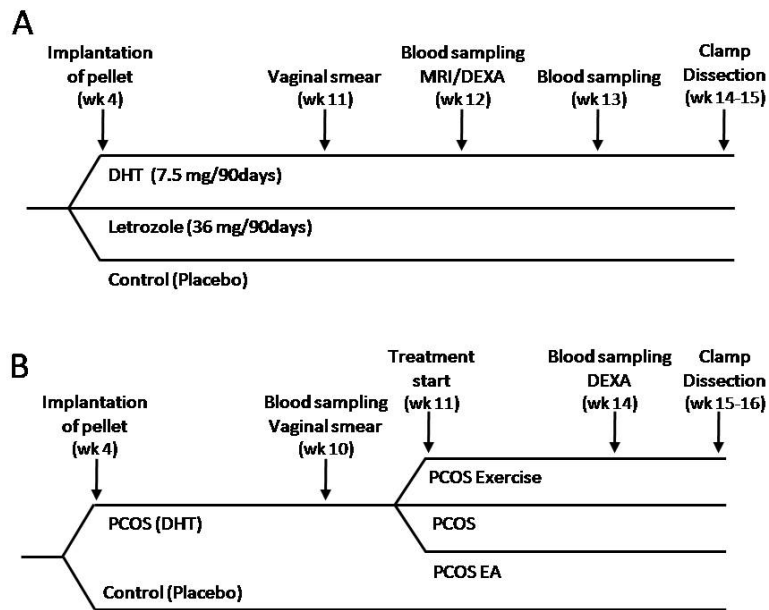


Figure 8. A) Study design and time axis of paper I. B) Study design and time axis of papers II and III. (DHT – dihydrotestosterone, wk – week of age, DEXA – dual-energy X-ray absorptiometry, MRI – magnetic resonance imaging)

2.3 Treatment (papers II and III)

Treatment was started at 69 days of age, 7 weeks after the start of DHT exposure. The rats in the PCOS group were randomly assigned to: i) PCOS, ii) PCOS exercise, and iii) PCOS EA. Low-frequency EA was given to conscious rats every second weekday for 4-5 wk (12-14 treatments in total). Treatment duration was 15 min in wk 1, 20 min in wk 2-3, and 25 min thereafter. Stimulation acupuncture points were in the rectus

abdominis [i.e. stomach (ST) 29] and in the triceps surae muscles [i.e. spleen (SP) 6] bilaterally, in somatic segments corresponding to ovarian innervation (i.e. from spinal levels T10 to L2 and at sacral level).

The points were electrically stimulated with a low frequency of 2 Hz with burst pulses (a burst length of 0.1 sec and a burst frequency of 80 Hz). Intensity was adjusted until local muscle contractions were seen, confirming activation of muscle-nerve afferents (A δ fibers and possibly C fibers). Before needle insertion, the rats were lightly anesthetized with isoflurane. All the needles were inserted by one specific investigator. After needle insertion, the rats were placed in a fabric harness and suspended above the desk during EA treatment.

Rats in the PCOS exercise group had free access to a wheel in their own cage and were allowed to exercise voluntarily for 4-5 weeks. This is a model of physical exercise that has been shown to be non-stressful, as the rats are free to move into the wheel at will and are not forced to run.³⁵⁵ Customized computer software registered all wheel rotations.

Three times a week, the rats in the PCOS group and the PCOS exercise group were anesthetized, suspended in a harness and handled in the same way as rats in the PCOS EA group but without needle insertion and electrical stimulation. All rats were conscious during the handling/treatment procedure.

To avoid any possible acute effects of EA or physical exercise, the running wheels were locked, and no EA treatment was performed for 24 h before examinations and blood sampling.

3 Human study (Paper IV)

3.1 Subjects

Potential participants with PCOS and controls were recruited by advertising in local newspapers and in frequently visited places in the community. All potential participants were asked to describe their medical history. They underwent a gynecological examination and two-dimensional transvaginal ultrasonography to investigate ovarian morphology.

Potential controls were excluded if they had evidence of PCO, menstrual irregularities (cycles < 28 days or > 35 days), excessive acne or hirsutism.

Seventy-four women with PCOS and 30 controls were included in the study. The controls were pair-matched with 30 of the women with PCOS according to age (± 5 years) and BMI (± 2 kg/m²).

The PCOS inclusion criteria were:

- PCO morphology – at least one ovary with 12 or more 2-9 mm follicles and/or increased volume (>10 ml) with:
 - Clinical signs of hyperandrogenism – a Ferriman Gallwey hirsutism score ≥ 8 ³⁵⁶ and/or acne: (*Do you have bad acne, Yes/No?*) and/or
 - Oligo/amenorrhea⁶ – Oligomenorrhea: (intermenstrual interval >35 days with less than eight menstrual bleedings in the past year) or amenorrhea (absent menstrual bleeding or no menstrual bleeding in the past 90 days).

The exclusion criteria for all women were:

- Pharmacological treatment in the preceding 12 weeks
- Breast feeding in the preceding 24 weeks
- Cardiovascular disease
- Diabetes
- Other endocrine disorders, such as congenital adrenal hyperplasia, Cushing's syndrome or androgen-secreting tumors

3.2 Samples

All participants were examined and blood and adipose tissue samples were taken in the morning after an overnight fast. Controls were examined during the early follicular phase (day 1-7 of the menstrual cycle). Most of the women with PCOS had oligo/amenorrhea, which is why the date of examination was set without reference to the menstrual cycle.

LPL activity, macrophage density and adipocyte size were determined in aliquots of adipose tissue biopsies taken from the abdominal subcutaneous depot of all participants.

4 Summary of the methods (papers I-IV)

The methods used in this thesis (Table 2) are described in detail in the Material and Methods sections of the individual papers, while more general comments are made below.

Table 2. Summary of the methods used in this thesis.

Methods	Experimental studies			Human study
	Paper I	Paper II	Paper III	Paper IV
Estrous cyclicity	■		■	
Ovarian morphology	■		■	
Assessment of body composition				
Dissection/anthropometry	■	■		■
DEXA	■	■		
MRI	■			■
Clamp	■	■		■
Adipocyte size	■	■		■
Real-time RT-PCR		■	■	
LPL-activity				■
Immunohistochemistry				■
Analytical methods				
Immunoassays	■	■		■
MS				■

DEXA – dual-energy X-ray absorptiometry, MRI – magnetic resonance imaging, RT-PCR, – reverse transcriptase PCR, LPL – lipoprotein lipase, MS – mass spectrometry.

5 Estrous cyclicity and ovarian morphology (papers I-III)

5.1 Estrous cyclicity (papers I, II and III)

Estrous cyclicity was monitored daily by vaginal smear from 10 weeks (papers II and III) or 11 weeks (paper I) of age to the end of each experiment. The normal duration of the estrous cycle in rats is 4-5 days, and it consists of four stages: proestrus, estrus, metestrus and diestrus.³⁵⁷ Each stage was determined by microscopic analysis of the predominant cell type. Proestrus is characterized by the presence of nucleated epithelial cells, estrus by anucleate cornified cells, metestrus by leukocytes, cornified and epithelial cells, while diestrus is characterized by the presence of a large number of leukocytes.

5.2 Ovarian morphology (papers I and III)

Ovarian morphology was evaluated after sectioning and staining with hematoxylin and eosin. Each slide was scanned and analyzed with virtual microscopy software (ImageScope, Aperio Technologies) by two persons blinded to the origin of the sections. The area of the ovary was measured with a calibrated scale tool. Antral follicles, defined as follicles with an antrum, were counted and classified as atretic or healthy. A cystic follicle was defined as a large fluid filled cyst with an attenuated granulosa layer and a thickened theca interna layer. The thickness of the follicular wall was measured. Corpora lutea were noted but not counted. All analyses were done by two investigators.

6 Assessment of body composition (Papers I-IV)

The anthropometric measurements in paper IV included body height, body weight, waist and hip circumferences and sagittal diameter. BMI [body weight (kg)/body height² (m²)] and waist-to-hip ratio (WHR = waist circumference/hip circumference) were also calculated. These measurements give information of the degree of adiposity and the adipose tissue distribution but they cannot distinguish between abdominal subcutaneous and abdominal visceral fat. Therefore, MRI was used in humans (paper IV) and in rats for a more detailed analysis of body composition (paper I).

In addition to continuous body weight measurement, individual fat depots (inguinal, parametrial, mesenteric and retroperitoneal) were dissected in the animal studies (papers I-II). DEXA was used to estimate whole body composition in the animal studies.

6.1 DEXA (papers I and II)

DEXA was originally intended for bone density analysis. However, it has become a widely used non-invasive technique for the investigation of body composition in humans and animals. The technique is based on the physics principle that X-rays of different energies are attenuated differently when passing through the body. This allows the assessment of total bone mineral content (BMC), lean body mass (LBM) and fat mass. It is important to note that DEXA measurements of fat summate all fat elements and do not directly measure the amount of adipose tissue. This method is also limited by the fact that it is not possible to quantify specific tissues.³⁵⁸

6.2 MRI (papers I and IV)

MRI is a technique that provides good soft tissue contrast without exposing the subject to any harmful ionizing radiation.³⁵⁸ MRI uses a powerful magnetic field, radio waves and a computer to generate detailed images of tissue, bone and other internal organs from any orientation. Adipose tissue can be distinguished easily in MR images as bright areas of high signal intensity that contrast clearly with other tissues. MRI is a validated method for accurately measuring adipose tissue mass in the subcutaneous and intra-abdominal compartments.³⁵⁹

Manual analysis of adipose tissue from MRI data is time-consuming and subject to investigator bias. In paper IV we used a fully automated algorithm for the quantification of total, visceral and subcutaneous adipose tissue from axial abdominal MRI data.³⁶⁰ Volumes of total, visceral and subcutaneous adipose tissue were quantified from sixteen sequential 10 mm sections centered on the L4-L5 interface.

MRI was also used for rat body fat distribution analysis in paper I. The distribution of subcutaneous and intra-abdominal fat was determined by analyzing the seventh axial slice from the most caudal part of the kidney, and a coronal slice.

7 Euglycemic-hyperinsulinemic clamp (Papers I, II and III)

Insulin sensitivity can be estimated by several methods, all based on the relationship between glucose and insulin. The gold standard method of evaluating insulin sensitivity *in vivo* is considered to be the euglycemic-hyperinsulinemic clamp technique. The clamp technique measures the steady state amount of glucose metabolized per unit of body weight during whole-body exposure to a predetermined amount of insulin, while maintaining the plasma glucose with the euglycemic range.³⁶¹ Plasma insulin concentration is acutely increased and maintained by a continuous infusion of insulin. Blood glucose levels are “clamped” at basal levels (euglycemic levels) by a simultaneous variable glucose infusion. Because endogenous hepatic glucose production is inhibited at high insulin concentrations, the amount of glucose infused per unit of time to maintain euglycemia reflects the amount of glucose metabolized in the peripheral tissues and is therefore an indirect index of the sensitivity of tissue to exogenous insulin. The insulin sensitivity index obtained by the clamp technique is basically the mean glucose infusion rate at steady state and termed glucose infusion rate (GIR) or glucose disposal rate (GDR or M), which is normalized to body weight (mg/kg x min). The degree of insulin resistance is inversely proportional to the glucose uptake by tissues during the procedure. Accordingly, an insulin-resistant subject requires less glucose to maintain euglycemia and has therefore a low GIR/GDR value.

However, because this technique is invasive and time-consuming, it is difficult to carry out in routine clinical practice or in large epidemiological studies and its use is limited to research laboratories. Consequently, several alternative tests have been developed. These include simple indexes obtained from fasting plasma glucose and insulin, such as homeostasis model assessment (HOMA)³⁶² and the quantitative insulin sensitivity check index (QUICKI).³⁶³ Each of these tests has been shown to correlate reasonably well with the euglycemic-hyperinsulinemic clamp technique.³⁶⁴ In paper IV, not all subjects underwent the clamp procedure, and a predicted GDR value was calculated based on the correlation between logHOMA and clamp-derived GDR values.

8 Adipocyte size determination (Papers I, II and IV)

There are several methods for determining adipocyte size, each with its advantages and disadvantages. Traditionally, adipocyte size diameter in a cell suspension, prepared by collagenase digestion of fresh tissue, is determined using a microscope and an ocular scale. Other methods include microscopic measurement of conventional histological preparations of adipose tissue. However, the latter method may be problematic due to the fragility of adipocytes and potential distortion caused by sectioning.³⁶⁵ Computerized image analysis for determination of adipocyte size, following collagenase digestion, enables objective, rapid and accurate of thousands of adipocytes, while conventional methods often are restricted to evaluation of hundreds of cells.³⁶⁶ Another advantage is that images of cell preparations can also be stored for future analysis and reference. One limitation of collagenase digestion is potential rupture of large adipocytes resulting in lipid droplets in the preparations and, in case of pronounced breakage, incorrect size determinations. Using precautions such as siliconized glassware and no centrifugation of the cells, the proportion of lipid droplets in the cell suspension is usually low. Additionally, using computerized image analysis, identified lipid droplets in the images can easily be excluded from the calculations. However, these lipid droplets need to be excluded manually and it may sometimes be difficult to tell the difference between small intact fat cells and small lipid droplets.

Adipocytes were isolated by digesting adipose tissue with collagenase at 37 °C in a gently shaking water bath. The stromal-vascular fraction was separated from the adipocytes by filtration through a 250µm-pore nylon mesh. The adipocytes were washed three times and suspended in fresh medium. The cell suspension was placed between a siliconized glass slide and a coverslip and transferred to a microscope. Nine random visual fields were photographed.

Adipocyte diameter was determined by computerized image analysis using KS400 Software (Carl Zeiss) or Leica software (Leica QWin V3, Leica Microsystems). Relevant surface areas were measured automatically, and diameters of the corresponding circles calculated. Uniform microspheres (diameter, 98 μm) served as a reference. The volume of the adipocytes was calculated from the diameter using Goldrick's formula ($\pi d [3(\text{S.D.})^2 + d^2]/6$), where d is mean adipocyte diameter and SD is the standard deviation of adipocyte diameter.³⁶⁷

9 Real time RT-PCR (Papers II and III)

Real-time reverse transcriptase (RT)-PCR is thought to be the most sensitive method for the detection and quantification of specific messenger RNAs (mRNA). This technique is sensitive enough to measure the amount of RNA in a single cell.

This method is based on the general principle of PCR, by which the target is amplified during the process. However, real-time RT-PCR is a "two-step" method for quantifying mRNA. Firstly, total RNA is reverse transcribed into complementary DNA (cDNA), as the RNA molecule is not stable enough for PCR. In the second step, the PCR amplifies the cDNA using primers and a probe complementary to the target sequence. The probe is labeled with a reporter dye at the 5' end and a quencher dye at the 3' end. During PCR, the probe anneals specifically between the primers to an internal region of the PCR product, and a DNA polymerase carries out the extension of the primers and replicates the template. The 5' exonuclease activity of the enzyme *Taq* DNA polymerase cleaves the reporter dye on the probe. The cleavage terminates the activity of a quencher dye, and the reporter dye starts to emit fluorescence which increases in each cycle proportional to the amount of probe cleavage and is monitored in real-time. The cycle at which the fluorescence from a sample crosses a fixed detection threshold is called the cycle threshold, C_T .

We used TaqMan® low density array (LDA) cards. These are 384-well micro fluidic cards that enable the simultaneous detection and quantification of several genes. The LDA card allows for 1-8 samples to be run in parallel against 12-384 targets. The wells are pre-loaded with probes and primers for the genes under study. We designed LDA cards in 24-format, including the target genes being studied and four putative reference genes.

Two different methods are commonly used to quantify the results obtained by real-time RT-PCR – the standard curve method and the comparative threshold method. Our LDA card made use of the latter method.³⁶⁸ With the comparative C_T method the amount of target is normalized to an endogenous reference (housekeeping) gene and relative to a calibrator/control, and is given by $2^{-\Delta\Delta C_T}$.³⁶⁹ The C_T values of both the

control and the samples being studied are first normalized to an appropriate endogenous reference gene/s [$\Delta C_T = C_T(\text{target}) - C_T(\text{reference})$]. The reference gene is an internal control that corrects for minor differences in the amount of input RNA or reverse transcription efficiency between samples. An optimal reference gene should be expressed at a constant level in different tissues of an organism and at all stages of development, and it should not be affected by the experimental setup.³⁷⁰ Normalization can involve one or several reference genes. We used the Normfinder algorithm to identify the reference gene with lowest variability.³⁷¹ Secondly, ΔC_T is converted to relative quantities, resulting in a $\Delta\Delta C_T$ value showing the cycle threshold differences after normalizing to a reference and to a control/calibrator [$\Delta\Delta C_T = \Delta C_T(\text{sample}) - \Delta C_T(\text{control})$]. This control/calibrator can be any sample, e.g. a real untreated control, or the sample with the highest expression. Thirdly, $\Delta\Delta C_T$ is converted to fold changes, $2^{-\Delta\Delta C_T}$.

Although real-time RT-PCR is considered to be the most sensitive technique for quantification of mRNA, there remain a number of problems associated with each step of the procedure, such as RNA quality, efficiency of the cDNA synthesis and data analysis.³⁷²

10 LPL-activity in adipose tissue (Paper IV)

LPL activity was measured in subcutaneous adipose tissue from women with PCOS and controls fasting over night. LPL activity was measured as described previously.³⁷³ After termination of lipolysis by addition of organic solvents, the fatty acids were extracted and counted for radioactivity. Incubation conditions (sample volume and/or incubation time) were adapted to give results in the linear range of the assay. Care was taken to analyze samples from matched pairs in the same assay to avoid effects of interassay variation. One mU of lipase activity represents one nmol of fatty acids released per minute.

11 Immunohistochemistry – macrophage density (Paper IV)

Macrophage investigation involved immunohistochemical staining of subcutaneous adipose tissue biopsies. Adipose tissue was fixed with neutral-buffered formalin, embedded in paraffin and sectioned. The sections were incubated with a monoclonal antibody against human CD68 to detect macrophages. The sections were counterstained with hematoxylin to visualize cellularity and then mounted. Tissue slides were scanned with a digital slide scanner (Mirax Desk, Zeiss) and analyzed using software (Mirax Viewer, Zeiss). The number of macrophages (identified as immunoreactive CD68+ cells) and the number of CLS (defined as an adipocyte

surrounded by at least three macrophages¹⁹⁸) within intact adipose tissue sections were counted and normalized for analyzed section area. The investigator was blind to the origin of the preparations.

12 Analytical methods (Papers I, II and IV)

The plasma/serum concentrations of analytes were determined with different immunoassays and mass spectrometry (Table 3).

Table 3. Summary of the analyses in each paper.

Analyte	Experimental studies		Human study
	Paper I	Paper II	Paper IV
Progesterone	IA		
Testosterone	IA		MS
17 β -estradiol	IA		MS
SHBG			IA
Insulin	IA	IA	IA
Glucose			IA
Corticosterone		IA	
IGF-1		IA	
Leptin	IA	IA	
Adiponectin			IA
SAA			IA
hs-CRP			IA
Glycerol			CM
Lipid profile	CM		

IA – immunoassay, MS – mass spectrometry, CM – colorimetric method, SHBG – sex steroid binding globulin, IGF-1 – insulin-like growth factor-1, SAA – serum amyloid A, hs-CRP – high-sensitivity C-reactive protein

12.1 Immunoassays

An immunoassay is a biochemical test that measures the concentration of a substance in a biological liquid, using the reaction of an antibody to its antigen. The assay takes advantage of the specific binding of an antibody to its antigen. Both the presence of antigen and antibodies can be measured, and this can be achieved by a variety of

methods by which either the antigen or antibody is labeled. The labels may consist of an enzyme (enzyme immunoassay [EIA]), radioisotopes (RIA), magnetic labels, or fluorescence. Reagents for direct immunoassays are obtained commercially in the form of kits and are therefore convenient, simple, quick and relatively inexpensive.

12.2 Mass spectrometry (paper IV)

The gas chromatography-mass spectrometry (GC-MS) technique was used in paper IV to measure serum testosterone and 17 β -estradiol. This method combines the power of gas chromatography to separate steroid hormones with the high-specificity and sensitivity of the mass spectrometer for quantifying a steroid. It provides highly reliable results. However, mass spectrometry assays are very expensive and they require a highly trained technician.

Free testosterone and free 17 β -estradiol were calculated using the method described by Vermeulen et al.³⁷⁴ and Van den Beld et al.,³⁷⁵ using the total concentrations of testosterone, estradiol, as measured by GC-MS and SHBG as measured by immunoassay, and assuming a fixed albumin concentration of 43 g/liter. Testosterone, 17 β -estradiol and calculated free testosterone in 31 controls and 74 PCOS women have been published.³⁷⁶

13 Statistical analyses (Papers I-IV)

Most statistical analyses were done using SPSS statistical software (SPSS inc., Chicago, IL, USA). Results are expressed as mean \pm standard error of the mean (SEM) in papers I-III and as mean \pm standard deviation (SD) in paper IV. $P < 0.05$ was considered significant.

13.1 Animal Studies (papers I-III)

Body weight changes were analyzed by repeated measures ANOVA (paper I) or repeated measures Friedman test (Paper II). In paper I, comparisons between groups were done by one-way ANOVA followed by Dunnett's *post hoc* test. In papers II and III, comparisons between groups were done with the Kruskal-Wallis test, and if significant, a Mann-Whitney *U*-test was performed between individual groups. Correlation analyses were done by linear regression (paper I) or the Spearman rank correlation coefficient (Rs) in bivariate analyses (paper II).

Adipocyte size distributions (paper I) were compared by using two-sample Kolmogorov-Smirnov statistics.³⁷⁷ An exact P-value for the comparison of the two groups was calculated through permutations. For these comparisons, statistical calculations were made using the R language (<http://www.R-project.org>).

13.2 Human study (paper IV)

We pair-matched 30 controls to 30 of the 74 women with PCOS. We carried out a specific analysis to study the size of confidence intervals with the aim of finding out whether the matching variables were important. We concluded that paired analysis was more powerful than un-paired analysis in almost every case, including the PCOS group. We therefore used paired Student's t test for the paired comparisons between the BMI and age-matched cases and controls. Multiple linear regression analysis was done to identify the relative independent determinants of insulin sensitivity in women with PCOS ($n=74$), with GDR as the dependent variable and MRI-estimated abdominal adipose tissue volumes, anthropometry, adipose tissue-related variables, and sex steroid-related variables as covariates. All variables were skewed, except age, height and adipocyte volume, and underwent transformation before statistical analysis. We applied the transformation $\Phi^{-1}(F(x))$ to transform continuous variables to normally distributed variables, where Φ^{-1} was the inverse of the standardized normal distribution function and F was the empirical distribution function. That transformation yielded an almost perfect normal distribution, which is not the case for logarithm transformation.

SUMMARY OF RESULTS

1 Paper I

1.1 Body composition and metabolic features

The continuous exposure of both DHT and letrozole from prepubertal age onwards resulted in a significant increase in body weight compared to control rats. In rats given letrozole, the change in body weight took place without any other major alteration in body composition. In contrast, the increased body weight seen in DHT-exposed rats was accompanied by an increase in body fat as determined by DEXA and an increase in intra-abdominal (parametrial, mesenteric and retroperitoneal) and subcutaneous (inguinal) adipose tissue depots in relation to body weight. Along with the differences in adiposity, rats given DHT developed larger mesenteric adipocytes and increased plasma leptin levels, whilst the levels of these parameters in rats given letrozole were no different to those found in controls. Fat distribution was also illustrated by MRI.

Whole-body insulin sensitivity, determined by euglycemic clamp, was found to be lower in rats given DHT than in controls, while no difference was found between rats given letrozole and controls. Lipid profiles (plasma concentrations of total cholesterol, triglycerides, FFA and high-density lipoprotein cholesterol) were similar in all groups.

1.2 Estrous cyclicity and ovarian morphology

Letrozole-exposed rats were completely acyclic, while DHT-exposed rats had irregular cycles, as determined by daily vaginal smears. Letrozole-exposed rats had enlarged ovaries with large atretic follicles (cysts) located in the periphery. Their follicles had a thickened wall characterized by a hypertrophic/hyperplastic theca interna cell layer and a relative thin granulosa cell layer. In some follicles, the inner wall in contact with cystic fluid was vascularized and had a scattering of luteinized granulosa cells. DHT-exposed rats had smaller ovaries with large atretic antral follicles. The follicular wall was thickened due to a hypertrophic/hyperplastic theca interna cell layer, while the granulosa cell layer was thinner, with the occasional apoptic granulosa cell, indicating atresia. As suggested by the cycle disturbances and abnormal ovarian morphology, DHT-exposed and letrozole-exposed rats had reduced plasma progesterone, and the latter also had increased plasma testosterone. Plasma 17β -estradiol was unaltered between groups. This lack of difference may be due to the low levels that normally occur in the estrus phase of regularly cycling rats.

2 Papers II and III

Plasma corticosterone levels and adrenal gland mass were lower in DHT-exposed rats than in controls. Four weeks of either exercise or EA treatment did not affect either corticosterone levels or adrenal mass, indicating that the treatments were not stressful to the rats.

2.1 *Body composition and metabolic features*

Body weight gain induced by continuous prepubertal administration of DHT was reversed by voluntary physical exercise over 4-5 weeks. Low-frequency EA treatment had no effect on body weight compared to untreated DHT-exposed rats. The exercise-induced change in body weight was mirrored by reduced total body fat as measured by DEXA, weight of individual fat depots, mean mesenteric adipocyte size and plasma leptin, compared to untreated DHT-exposed rats. These parameters were not affected by EA. Both exercise and EA improved insulin resistance induced by DHT. The fall in insulin-like growth factor-1 (IGF-1) levels in DHT-exposed rats was reversed by EA treatment, but not by exercise.

2.2 *Estrous cyclicity and ovarian morphology*

Fresh corpora lutea, an indication of ovulation, were observed in 45% of the DHT-exposed rats treated with EA, but not in the exercise group. Neither EA nor exercise had any effect on ovarian size. An increased proportion of atretic antral follicles with a thickened theca interna cell layer was seen in DHT-exposed rats compared to controls, which was partly reversed by both EA and exercise.

2.3 *Gene expression analysis in mesenteric fat*

Gene expression analyses in mesenteric adipose tissue demonstrated increased mRNA expression of leptin (*Lep*), interleukin 6 (*Il6*), beta 3 adrenergic receptor (*Adrb3*), nerve growth factor (*Ngf*), neuropeptide Y (*Npy*), and decreased expression of uncoupling protein 2 (*Ucp2*) in PCOS (DHT) rats. EA was able to normalize the expression of *Lep*, *Ucp2*, *Adrb3*, *Ngf* and *Npy*, whereas exercise normalized adipose tissue *Lep*, *Il6*, *Ngf* and *Npy* expression. In addition, EA also reduced the mRNA expression levels of the androgen receptor (*Ar*) and peroxisome proliferator-activated receptor gamma (*Pparg*), even though the expression of these genes was not significantly different in untreated DHT rats and controls. Gene expression is summarized in Table 4.

Table 4. Summary of gene expression in mesenteric adipose tissue in PCOS (DHT-exposed) *vs.* controls and in exercise and EA *vs.* no treatment in PCOS.

Gene ^A	PCOS <i>vs.</i> Controls	Exercise <i>vs.</i> untreated PCOS	EA <i>vs.</i> untreated PCOS
<i>Lep</i>	↑	↓	↓
<i>Il6</i>	↑	↓	=
<i>Tnf</i>	=	=	=
<i>Pparg</i>	=	=	↓
<i>Ucp2</i>	↓	=	↑
<i>Adrb3</i>	↑	=	↓
<i>Ar</i>	=	=	↓
<i>Ngf</i>	↑	↓	↓
<i>Ngfr</i>	=	=	=
<i>Npy</i>	↑	↓	↓

^AGene nomenclature/names according to Rat Genome database (RGD) (<http://rgd.mcw.edu>). *Lep* – leptin, *Il6* – interleukin 6, *Tnf* – tumor necrosis factor, *Pparg* – peroxisome proliferator-activated receptor gamma, *Ucp2* – uncoupling protein 2, *Adrb3* – beta 3 adrenergic receptor, *Ar* – androgen receptor, *Ngf* – nerve growth factor, *Npy* – neuropeptide Y

3 Paper IV

3.1 Paired comparisons

Women with PCOS had markedly higher levels of plasma testosterone, free testosterone and 17 β -estradiol, and reduced levels of plasma SHBG compared to controls. Women with PCOS also displayed reduced insulin sensitivity as determined by the clamp procedure. There were no differences in body composition and adipose tissue distribution between the groups, as measured by the anthropometric variables weight, height, waist and hip circumference, WHR, and sagittal diameter. Nor were there any differences in abdominal total, subcutaneous and visceral adipose tissue volumes as measured by MRI. Mean abdominal subcutaneous adipocyte size was increased in women with PCOS compared to controls. Serum levels of glycerol, an indirect marker of *in vivo* lipolytic activity, was similar in both groups, while women with PCOS had lower adipose tissue LPL activity of borderline statistical significance. Adiponectin is mainly produced in adipocytes and so is SAA under non-acute phase conditions. Serum adiponectin was lower in women with PCOS, while the serum concentration of SAA was similar in both groups. Adipose tissue macrophage density,

number of CD68-positive cells normalized for section area, was also similar in both groups.

3.2 Factors associated with insulin sensitivity in women with PCOS

Multiple linear regression (stepwise) was performed to identify factors associated with insulin sensitivity (clamp-derived) in women with PCOS. The independent variables included age, height, weight, BMI, waist circumference, hip circumference, WHR, sagittal diameter, adipose tissue volumes, adipocyte volume, adipose tissue LPL activity and macrophage density, as well as serum levels of testosterone, SHBG, free testosterone, free estradiol, glycerol, adiponectin and SAA. As neither adipose tissue volumes nor macrophage density contributed significantly to the models, these variables were excluded from the final analysis to allow the inclusion of a greater number of women with PCOS. Adipocyte volume along with serum adiponectin and waist circumference, were the factors strongest associated with insulin sensitivity. Adipocyte volume was the single factor strongest associated with insulin sensitivity in women with PCOS.

SUMMARY OF RESULTS

Table 5. Summary of the result presented in this thesis (papers I-IV).

	In general ^A		Paper I, II, III		Paper II and III	
	PCOS vs. controls	PCOS vs. controls	Letrozole vs. controls	DHT vs. controls	Exercise vs. untreated PCOS	EA vs. untreated PCOS
BMI/body weight	↑	Matched	↑	↑	↓	=
Obesity	↑	=	=	↑	↓	=
Abdominal obesity	↑	=	=	↑	↓	=
Adipocyte size	↑/= (sc)	↑ (sc)	= (mes)	↑ (mes)	↓ (mes)	= (mes)
Insulin sensitivity	↓	↓	=	↓	↑	↑
Ovarian size/weight	↑	↑(incl. criteria)	↑	↓	=	=
An/oligo-ovulation	Yes	Yes	Yes	Yes	Partly improved	Partly improved
Number of follicles/cysts	↑	↑ (incl. criteria)	↑	↑	↓ atretic follicles	↓ atretic follicles
Theca layer	↑	N/A	↑	↑	↓	↓
Plasma Leptin	=	N/A	=	↑	↓	=
Plasma IGF-1	↑/=	N/A	N/A	↑	=	↓
Plasma Adiponectin	↓	↓	N/A	N/A	N/A	N/A
Dyslipidemia	Yes	N/A	=	=	N/A	N/A
Testosterone	↑	↑	↑	=	N/A	N/A
Progesterone	↓ (if anov)	N/A	↓	↓	N/A	N/A
Estradiol	↑	↑	=	=	N/A	N/A

^A General interpretation of available scientific literature on PCOS. BMI – body mass index, sc – subcutaneous adipose tissue, mes – mesenteric adipose tissue, N/A – not available, IGF-1 – insulin-like growth factor-1, anov – anovulatory

DISCUSSION

The main findings of the present thesis

- Androgen exposure from prepubertal age induces a phenotype similar to human PCOS in adult female rats, including irregular cycles, PCO-like morphology, insulin resistance and increased fat accumulation.
- Women with PCOS display hyperandrogenemia, insulin resistance, low blood adiponectin levels and enlarged subcutaneous abdominal adipocytes, although their anthropometry and abdominal adipose tissue volumes (total, subcutaneous and visceral) are indistinguishable from controls matched for age and BMI.
- Adipocyte size, circulating adiponectin and waist circumference, but not circulating sex steroids, may be central factors in the pathogenesis of insulin resistance in women with PCOS.
- Both low-frequency EA and physical exercise improve insulin resistance and rectify the altered adipose tissue expression of genes related to insulin resistance, obesity, inflammation and sympathetic activity. In contrast to exercise, EA exerts its beneficial effects without affecting adiposity and adipose tissue cellularity.

Androgens and PCOS-related signs and symptoms

Insulin resistance, with compensatory hyperinsulinemia, plays a major role in the metabolic abnormalities associated with PCOS. Although, not all women with PCOS are insulin-resistant or develop compensatory hyperinsulinemia, implying that these features are not essential to develop PCOS. However, androgen excess is the principal biochemical abnormality in women with PCOS, and the clinical manifestations of hyperandrogenemia usually appear around puberty.^{349,350} There is also evidence that androgens contribute significantly to the development of metabolic disturbances.⁷⁹ This suggests that a primary defect in androgen metabolism is the intrinsic, fundamental factor in the pathogenesis of PCOS.²⁶³

It is thought that peripubertal age is a sensitive period during which an insult can have pronounced and lifelong effects on both ovarian and metabolic function.^{285,349,351,352} Our aim was to induce a hyperandrogenic state by administering DHT or letrozole to female rats from prepubertal age onwards, so that we could investigate the resulting metabolic and ovarian abnormalities. Specific androgen receptor activation by continuous exposure of DHT produces insulin resistance in adult female rats,

confirming the ability of androgens to alter the mode of action of insulin. Indeed, several studies show that female rats exposed to exogenous androgens prenatally, neonatally or peri-pubertally develop insulin resistance.^{101,102,275,276,285,378} Other studies^{102,276} show that rats exposed to androgens become obese, and so we cannot be sure that insulin resistance is not merely a reflection of increased adiposity. In contrast, rats exposed to letrozole put on weight without exhibiting any change on body composition or whole-body insulin sensitivity.

Obesity in women is *per se* a condition of sex hormone imbalance. An increase in body weight is associated with increased androgen levels in both women with PCOS and in normal controls.^{185,379} In addition, androgens play an important role in the regulation of body fat distribution.³⁸⁰ Androgen excess in women is generally associated with abdominal obesity, which in turn is a critical determinant of obesity-related metabolic complications.³⁸⁰ The inverse association between plasma SHBG and abdominal obesity in women suggests that obesity aggravates androgen excess, which may partly be explained by hyperinsulinemia secondary to insulin resistance.^{138,379} However, adipose tissue serves as an intracrine source of androgens,¹⁸⁶ which may partly explain the positive correlation between circulating androgens and obesity in women.¹⁸⁵ Further evidence that excessive androgens in women may be a trigger factor for the development of insulin resistance and abdominal fat accumulation comes from studies in which women were treated with exogenous androgens.^{261,266-269} In contrast to many previous studies, we did not observe a difference in abdominal fat distribution or anthropometric variables between women with PCOS and controls matched for BMI and age. However, women with PCOS had reduced whole-body insulin sensitivity and hyperandrogenemia, suggesting that insulin resistance in women with PCOS is a feature that is partly independent of body weight and fat distribution.

The rats exposed to letrozole developed irregular cycles and enlarged ovaries of polycystic morphology similar to that seen in human PCOS, i.e. multiple cysts/follicles with thickened theca cell layers, located in the periphery. The rats exposed to DHT also displayed irregular cycles and an increased number of follicles with thickened theca cell layer, but their ovaries were smaller than those of control rats. In addition, the high proportion of atretic follicles in our anovulatory rat models of PCOS differs from human PCO, in which the proportion of atretic follicles is smaller.³⁸¹ The interpretation of ovarian abnormalities must be made in the context of the differences between rodents and humans in the ovulation cycle and in the formation and recruitment of ovarian follicles.

Adipose tissue and insulin resistance in PCOS

Androgens can exert direct effects on adipose tissue function and insulin sensitivity via adipocyte androgen receptors. The density of these receptors is higher in visceral fat than in subcutaneous fat.²⁶⁴ *In vitro* studies have shown that androgens counter the action of insulin in adipocytes.⁹⁸ Androgens also influence insulin sensitivity indirectly via effects on body fat distribution, lipid metabolism and adipokine release.^{265,379,382}

The data presented in this thesis show that women with PCOS and rats with DHT-induced PCOS have larger fat cells than controls, but this was not seen in letrozole exposed rats. Adipocyte size is an independent predictor of T2DM.^{241,242} The mechanisms by which enlarged fat cells contribute to insulin resistance and the development of T2DM are not completely understood. The pattern of adipokine secretion in enlarged fat cells differs from that seen in smaller adipocytes,²⁵⁰ and this may indirectly affect insulin action in other tissues. Similarly, and independent of BMI, women with PCOS have lower plasma levels of adiponectin, an adipokine with anti-diabetic properties that is almost exclusively produced in adipocytes.¹⁸¹ Moreover, rats with DHT-induced PCOS have higher plasma leptin, an adipokine that is almost exclusively produced by fat cells and which is associated with insulin resistance. The increased plasma leptin seen in these rats may be a reflection both of the increased fat cell size and increased body fat mass. Studies in women with PCOS have shown that they have similar plasma leptin levels as controls matched for BMI or weight,¹⁷⁸⁻¹⁸⁰ although other studies have shown higher leptin levels in women with the syndrome, independent of BMI.³⁸³

Studies of adipose tissue LPL activity in women with PCOS are few and inconsistent, with some showing lower and others showing similar activity compared to controls.^{231,232} Testosterone seems to have an inhibitory effect on adipose tissue LPL activity in human fat cells,³⁸⁴ which suggests that women with PCOS are likely to have decreased LPL activity in adipose tissue. However, androgens can be aromatized into estrogens, and therefore the circulating levels of sex steroids do not necessarily reflect the sex steroid composition within adipose tissue.³⁸² In the human arm of this thesis, we observed a substantial but statistically non-significant reduction in adipose tissue LPL activity in women with PCOS compared to matched controls. Low LPL activity in these women may also be a reflection of insulin resistance.³⁸⁵

Women with PCOS are thought to be insulin-resistant and to have an abdominal fat distribution independent of obesity. The typical android body fat distribution could partly explain the metabolic disturbances often observed in women with PCOS. However, women with PCOS in our study were insulin-resistant independently of BMI and with an adipose tissue distribution indistinguishable from controls, implying

that other factors must be involved. Sex hormone levels and adipose tissue related factors are plausible candidates. We therefore carried out a multiple linear regression analysis to identify independent factors associated with insulin sensitivity in women with PCOS. Notably, neither MRI-determined abdominal adipose tissue volumes nor sex steroid related variables were included in the best models. Instead, the adipose tissue characteristic adipocyte size, along with serum adiponectin and waist circumference (in part reflecting degree of abdominal fat accumulation), were found to be the strongest covariates. Taken together, this indicates that the adipose tissue in PCOS is characterized by disturbed cellularity and function, which may result in insulin resistance. A large waistline may further aggravate this disturbance.

As visceral adipose tissue is metabolically highly active and strongly involved in metabolic abnormalities such as insulin resistance, we chose to focus our experimental studies on the mesenteric fat depot. Gene expression analyses demonstrated that DHT-exposed rats have altered expression of genes related to insulin resistance, obesity, inflammation, and sympathetic activity. The DHT-exposed rats had a higher mRNA expression of *Lep* consistent with the higher circulating leptin levels compared to controls. Moreover, mRNA expression of the proinflammatory cytokine IL-6 was increased, while mRNA expression of *Ucp2*, which is thought to be involved in the pathogenesis of obesity,³⁸⁶ was decreased compared to controls.

It has been suggested that altered activity in the sympathetic nervous system contributes to the pathogenesis of PCOS.⁴⁰ Several PCOS-related factors are associated with increased sympathetic activity,⁴¹⁻⁴⁴ and indirect measurements have shown that women with PCOS have an increased sympathetic and a decreased parasympathetic component.⁴⁹⁻⁵¹ The suggestion of sympathetic nervous system involvement in the pathogenesis of PCOS is further supported by the finding of a greater density of catecholaminergic nerve fibers and enhanced ovarian NGF production in PCO.^{45,46,48} We were recently able to demonstrate, by direct intraneural recordings, that women with PCOS have increased sympathetic nerve activity, which correlated positively with testosterone levels.⁵² However, it remains unclear whether increased sympathetic activity causes PCOS or if it is a consequence of hyperandrogenemia. In our rats with DHT-induced PCOS, the mRNA expressions of several markers of sympathetic nervous system (*Ngf*, *Npy* and *Adrb3*) were increased in adipose tissue compared to controls. This lends further support to the theory that androgen excess contributes to the high sympathetic activity seen in women with PCOS.

Effects of EA and exercise on metabolic disturbances and ovarian dysfunction

Whatever the primary cause of PCOS, androgens and insulin create a vicious circle that must be broken. Improving insulin resistance and reducing serum insulin levels by treatment with insulin sensitizers often improves the symptoms of hyperandrogenemia in women with PCOS.³⁹ Similarly, anti-androgen treatment may improve insulin sensitivity in the same women.²⁷⁰⁻²⁷² Although pharmacological approaches are usually effective, they may have adverse effects. Lifestyle modification combined with exercise is a safe and inexpensive initial management for PCOS, especially in the overweight, as it improves reproductive function and metabolic profile.^{151,288,293,295,297,298} Indeed, repeated treatments with acupuncture has been shown to be a safe treatment option that improves endocrine and reproductive variables in women with PCOS and women with undefined ovulatory dysfunction.^{290,292,301}

Insulin resistance, which is a hallmark of PCOS, and which aggravates both endocrine and metabolic features, responded to EA and to exercise in rats with DHT-induced PCOS. The observed and expected reduction in body weight, adiposity and adipocyte size may partly explain the beneficial effect of exercise. However the beneficial effects of EA on insulin sensitivity occurred in the absence of any effect on adipose tissue mass or cellularity.

As skeletal muscle accounts for 85% of whole body insulin-stimulated glucose uptake,⁸⁰ one might assume that skeletal muscle is the only target tissue for glucose homeostasis. However, adipose tissue is now thought to play a central role in the determination of whole body insulin sensitivity.⁸¹ It is thought that adipose tissue can indirectly contribute to insulin action in other tissues by secretion of fatty acids, cytokines, and adipokines acting in an autocrine, paracrine and endocrine manner. The negative crosstalk is illustrated by co-culture studies with adipocytes, in which insulin resistance was induced in skeletal muscle cells.^{387,388} To evaluate the contribution of adipose tissue to the improved insulin sensitivity seen with EA and exercise, we investigated gene expression in mesenteric adipose tissue. Both plasma leptin and *Lep* mRNA expression in adipose tissue fell after physical exercise in rats with DHT-induced PCOS. Although no change was seen in circulating leptin levels after EA, mesenteric *Lep* mRNA expression fell. In addition, *Il6* was significantly downregulated by exercise, and a tendency towards reduction was also observed after EA. EA also partly reversed the DHT-induced reduction of *Ucp2* mRNA expression. It has been suggested that the abnormal release of adipokines promotes hyperandrogenism in PCOS by influencing ovarian and adrenal function directly, and indirectly via the induction of insulin resistance and hyperinsulinemia.²⁶³ Therefore, hypothetically,

these alterations of adipose tissue gene expression seen after exercise and EA may positively influence peripheral tissue and whole-body metabolism.

Both EA and exercise improved ovarian morphology in rats with DHT-induced PCOS, indicated by a lower proportion of atretic follicles and a thinner theca interna cell layer. In the EA group, fresh corpora lutea were observed, indicating recent ovulations. This is consistent with previously published findings of beneficial effects of exercise on ovarian morphology in a rat model of PCO induced by estradiol valerate.³⁸⁹ Additionally, we have recently shown that more intensive low-frequency EA (5 times/week compared to the 3 times/week used in papers II and III) improves estrous cyclicity in rats with DHT-induced PCOS.³⁹⁰

Androgens increase the expression of their own receptor. Although we did not observe any significant increase in *Ar* mRNA expression in adipose tissue in rats with DHT-induced PCOS, we observed a decrease in expression following EA treatment in the same rats. Similarly, frequent EA reduced DHT-induced elevation of hypothalamic AR and GnRH expression,³⁹⁰ which may represent a possible mechanism for the improvement induced by EA in the reproductive and endocrine function in both women with PCOS and rats with DHT-induced PCOS.^{290,292,301,390,391}

The sympathetic nervous system has been implicated in the control of ovarian function.^{320,321,392} The excessive ovarian NGF production observed in women with PCOS compared with controls lends further support to the theory that sympathetic hyperactivity has a role in ovarian dysfunction.⁴⁸ The beneficial effect of EA and exercise may therefore reflect their capacity to inhibit ovarian sympathetic neurons.

Furthermore, treatment with low-frequency EA *and* physical exercise reduced the expression of several markers of sympathetic activity in the DHT-induced rat model of PCOS. Similar reductions with EA and exercise have been observed in ovaries from rats with estradiol valerate-induced PCO.^{389,393,394} This suggests that a reduction of sympathetic activity may represent a possible mechanism by which EA and physical exercise exert their beneficial effects on both metabolic and ovarian features. Interestingly, we recently demonstrated that both low-frequency EA and physical exercise reduce high sympathetic nerve activity in women with PCOS,³⁰¹ providing further evidence that EA and exercise represent treatment options that may reduce the sympathetic activity associated with PCOS.

In summary, improvements in the expression of a variety of adipose tissue genes related to insulin resistance, obesity, inflammation and sympathetic activity may partly explain the beneficial effects of low-frequency EA and physical exercise on metabolic and ovarian disturbances in the rat models being studied.

CONCLUDING REMARKS

Although the etiology of PCOS is still poorly understood, androgens and insulin are thought to be two key factors in its pathogenesis. They are closely related, but the nature of their interaction has been a subject of debate since the association was first noted. Our experimental studies indicate that androgens probably play a central role in the pathogenesis of PCOS. Rat models of PCOS, induced by DHT or letrozole, highlight the close relationship between androgen excess and the development of the typical ovarian and/or metabolic disturbances.

It is widely believed that most women with PCOS are insulin-resistant and have an abdominal fat distribution even in the absence of obesity. The women with PCOS in our study displayed hyperandrogenemia and insulin resistance, but their anthropometrical variables and abdominal adipose tissue volumes were indistinguishable from controls. Therefore, insulin resistance in women with PCOS seems to depend on factors other than adipose tissue mass and adipose tissue distribution. We used sophisticated, gold standard methods (clamp, mass spectrometry and MRI) to measure these variables, making our results highly reliable. Furthermore, the women included in our study covered a wide range of BMI. Another recent study, which also used MRI for analyzing adipose tissue, concluded, as we do, that the adipose tissue distribution of women with PCOS is indistinguishable from that of BMI-matched controls, despite significant differences in insulin resistance.¹³⁷ The women with PCOS in our study had adipose tissue aberrations, including enlarged fat cells, reduced plasma adiponectin and, possibly, decreased adipose tissue LPL activity. Out of the variables investigated in the present study, enlarged adipocytes, low circulating adiponectin, and a large waistline, but not hyperandrogenemia, were the factors strongest associated with insulin resistance in the women with PCOS. These factors may therefore constitute part of the pathogenesis of insulin resistance in this group of patients.

It is important to break the vicious circle of PCOS, which is largely maintained by high levels of androgens and insulin. The observation that women with PCOS, and rats exposed to androgens, have signs of increased activity in the sympathetic nervous system suggests that therapies that reduce sympathetic activity may prove beneficial. The results of our experimental studies suggest that both low frequency EA and exercise may represent valuable non-pharmacological treatment alternatives with few side effects, with the potential to improve ovarian dysfunction and metabolic disturbances, and that they may act, at least in part, by reducing sympathetic activity.

FUTURE PERSPECTIVES

Although the results published in this thesis increase our understanding of PCOS, they inevitably lead to further questions and debate.

Our experimental studies suggest that androgen excess is an important pathophysiological factor in PCOS. Androgen exposure of female rats from peripubertal age results in PCO morphology as well as insulin resistance and adiposity, all disturbances typical of PCOS. However, translating experimental findings to human medicine should be done with caution. Clearly, further research regarding the pathogenesis of PCOS is needed. In addition, the relationship between androgen excess and insulin resistance in PCOS remains a chicken-and-egg mystery. Androgen excess may aggravate insulin resistance in skeletal muscle and adipose tissue of women with PCOS, and the resulting hyperinsulinemia may stimulate androgen production which in turn contributes to insulin resistance, i.e. a vicious circle may be established. It remains for future clinical studies to evaluate interventions aiming to lower androgen concentrations, or block androgen action, in young women with androgen excess to investigate if this approach protects against development of metabolic disorders such as insulin resistance and T2DM in later life.

In my thesis, I have shown that women with PCOS are indistinguishable from controls matched for age and BMI with respect to anthropometric variables and abdominal total, subcutaneous, and visceral adipose tissue volumes as determined by MRI. These results call into question the widely held belief that women with PCOS have an android/central type of fat distribution, different from the distribution in healthy controls. It is obvious that application of accurate methods for determination of the area, or volume, of separate tissue compartments is a prerequisite in this context. Future investigations of body composition in PCOS will be followed with interest.

We suggest that adipose tissue abnormalities in PCOS – enlarged fat cells and reduced adiponectin production – together with a large waistline, rather than androgen excess, are pivotal factors in the development and/or maintenance of insulin resistance in women with the syndrome. It would therefore be valuable to further investigate the adipose tissue in this group of women in terms of gene expression and release of adipokines. This could, for example, increase our knowledge of the contribution of adipose tissue to the proposed inflammatory status in PCOS. Also, little is known about the regulation of local androgen production in the adipose tissue of women, and the role of this production. Although samples are difficult to obtain, it would of course be interesting to also study the visceral fat depot of women with PCOS.

The beneficial effects of low frequency EA and physical exercise shown in our experimental studies suggest that these approaches may represent two therapeutic options for women with PCOS. There is increasing evidence that EA represents an alternative treatment for disturbances of endocrine and reproductive function, but the effects of EA on metabolic variables in women with PCOS are largely unknown. Randomized control studies are needed to compare the effects of low frequency EA and physical exercise with traditional pharmacological treatment.

ACKNOWLEDGMENTS

Resan fram till denna avhandling har definitivt inte varit någon ”one-man show”. Den hade inte existerat utan en mängd underbara människor som har stöttat och hjälpt mig samt förgyllt min tillvaro under dessa år. Jag vill särskilt tacka:

Min huvudhandledare **Elisabet (Lisa) Stener-Victorin**. Du har guidat mig genom forskningens fascinerande världen på ett exemplariskt och inspirerande sätt. Resan från början till slut har varit helt fantastisk tack vare dig. Du har alltid kommit med uppmuntrande ord precis när jag har behövt dem. Du är en stor förebild för mig och enligt mig den bästa handledaren man kan tänka sig.

Min bihandledare **Malin Lönn**, för att du alltid tagit dig tid och svarat på alla mina frågor. Jag värderar din enorma, detaljrika kunskap oerhört högt och är mycket glad att du har delgett mig den. Du är en person som värdesätter perfektionism, en egenskap jag beundrar .

Min bihandledare **Agneta Holmäng**. Tack vare att du trodde på mig efter mitt arbete för forskarskolan, så vågade ni ta risken och presentera ett doktorandprojekt för mig som jag utan tvekan tackade ja till. Tack för att du alltid tagit dig tid så fort jag har behövt din hjälp.

Julia Johansson, en språksam norrländska som förgyller min arbetsdag och inte allra minst våra gemensamma konferensresor. Du är precis den person jag kan tänkas knô med på kontoret och lösa alla världsproblem med.

Camilla Alexanderson, min trogna, gamla radarpartner. Du var mitt bollplank och guidade mig genom doktorandjungeln i början. Vi har rest på roliga konferensresor, skrattat, skvallrat, gråtit och haft givande vetenskapliga diskussioner tillsammans. Njut nu av livet som nybliven mamma.

Mina **medförfattare** som har gjort alla artiklarna möjliga. Speciellt tack till **Stefan Cajander** för din hjälp med tolkning av ovariemorfologi; **Joel Kullberg**, **Henrik Leonhardt**, **Mikael Hellström** och **Lars Lönn** för er MRI-expertis; **Eva Jennische** som gjorde det omöjliga möjligt – snitta och färga in fettbiopsier; **Gunilla Olivecrona** för all hjälp med LPL-analyserna; **Anders Odén** och **Theodor Lystig** för statistikhjälp; **Ingibjörg Jonsdottir** som gjorde det möjligt för våra råttor att motionera; och **Göran Holm** som såg till att de flesta analyserna för PCOS kvinnorna blev gjorda.

Alla härliga nuvarande och före detta **endokrinare** som tillhör/tillhört **John-Olov Janssons, Suzanne Dicksons, Agneta Holmängs, Håkan Billigs** och **Joakim Larssons** grupp. Tack var er är det en fröjd att komma till berget och jobba varje dag. Speciellt tack till **Robert Jakubowicz, Filip Cuklev**, och **Erik Schéle** som ger sitt manliga perspektiv på saker och ting i den kvinnodominerande miljön på fysiologen och som får mig att skratta även de dagar då jag egentligen inte har lust till det. **Louise Strandberg**, vi klarade det till slut – att både bli fru och doktor. Min gamla rumskamrat **Magda Taube**, tack för att du alltid finns till hands och har kloka svar på livets stora frågor. **Anna Benrick**, tack för sköna konferensresor och roligt sällskap, jag hoppas vi får tillfälle att träffas på svensk mark snart igen.

Lena Olofsson, som alltid lyckas gör det omöjliga möjligt avseende ekonomi och andra administrativa frågor.

Birgitta Odén, du alltid har ställt upp och dessutom hållit mig sällskap mitt bland alla fettbiopsier. Ditt brinnande intresse för miljön är inspirerande.

Britt-Mari Larsson, det var du som lärde mig allt jag behövde veta för att lyckas på EBM och i labbet.

Carola Gustavsson, min egna fettbiopsi-drottning. Utan dig så hade det inte funnits någon artikel IV.

Tjejerna som har gjort exjobb för mig, **Jessica, Emma, Kristy, Malin, Mirjana, Maja** och **Effie** – utan era extra händer så hade jag aldrig lyckats ro i hamn denna avhandling.

Mina fantastiska tjejer – **Kristin, Anna, Maria, Anna, Marie, Josefin, Camilla** och **Ellinor**, ni är de bästa vänner man kan tänka sig. Tack för ni förgyller mitt liv och har gjort en tid av frustration, stress och irritation ljusare genom att bara finnas till för mig.

Mats & Karin, Jerker & Maria, Jakob & Sofia, Gustav, Andreas & Sandra. Ett skönt gäng människor som jag alltid garanterat har kul med på ett okomplicerat och opretentiöst sätt. Tack för att ni finns och gör mitt liv roligare.

Eva och **Eric**, de underbaraste svärföräldrarna man kan tänka sig. Tack för er generositet och för att ni alltid ställer upp. Alltid tryggt att vet att ni finns en trappa upp och jag behöver hjälp med något.

Martin, bättre storebror kan man inte önska sig. Du sitter som en skyddsängel på min axel hela tiden. Tack för att du och **Ulrika** gett mig världens underbaraste brorsbarn – **Nike** och **Emil** – ni är solstrålarna i mitt liv.

ACKNOWLEDGMENTS

Mamma **Barbro** och pappa **Tomas**, ni är världens bästa föräldrar! Tack för att ni alltid har trott på mig och stöttat mig i alla de val jag har gjort i livet. Ni har länge undrat vad jag har gjort uppe på berget de senaste åren – ni har nu resultatet i era händer, vet dock inte om ni blir så mycket klokare av det ☺.

Min man **Kristian**. Tack för att du fulländar mitt liv. Tack för din villkorlösa kärlek. Tack för att jag får dela mitt liv med dig. Tack för att du gör mig till en bättre människa. Jag älskar dig över allt annat ♥.

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