Polycystic ovary syndrome

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

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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. Insulinokänsligheten 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 orgelbunden menstruation och äggstocksförändringar liknande de hos kvinnor med PCOS samt metabola rubbningar, såsom insulinokänslighet och fetma med förstorade 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 akupunkturnålarna, 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 äggstocksrubbningar. 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 äggstocksrubbningarna.

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

CONTENTS

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

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-1α	Macrophage inflammatory protein 1α
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
TNFα	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).

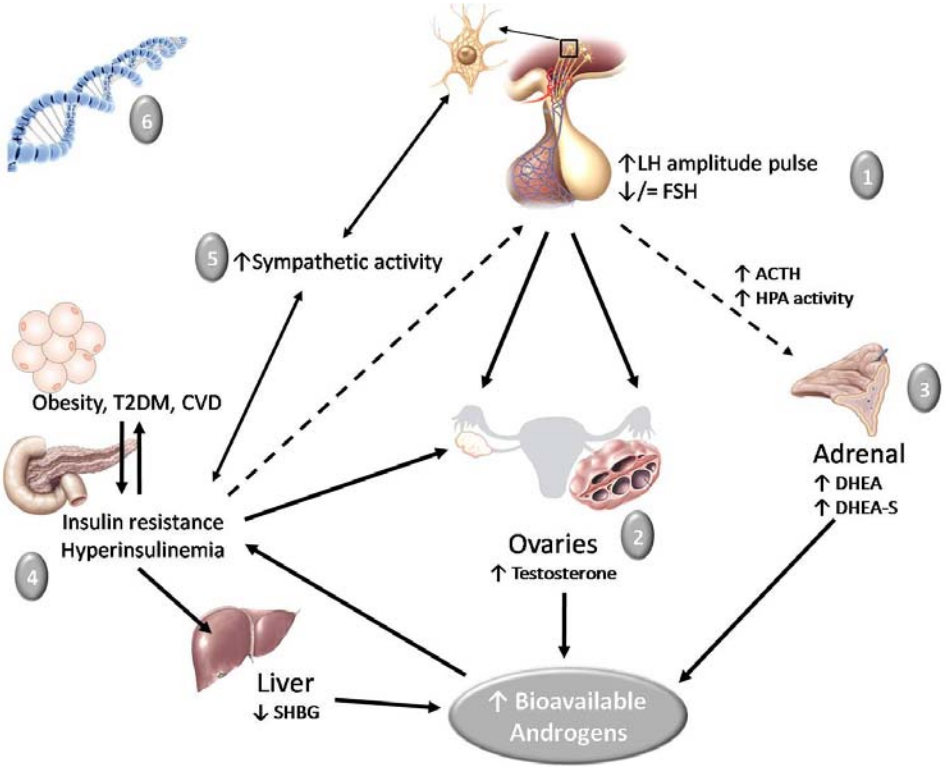

Figure 1. Several theories have been proposed to explain the pathogenesis of PCOS. 1) Neuroendocrine defects, leading to increased pulse frequency and amplitude of LH with relatively low FSH. 2) Intrinsic defects in ovarian androgen production. 3) Alteration in cortisol metabolism and impaired adrenal androgen production. 4) Insulin resistance with compensatory hyperinsulinemia which results in incrased 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.

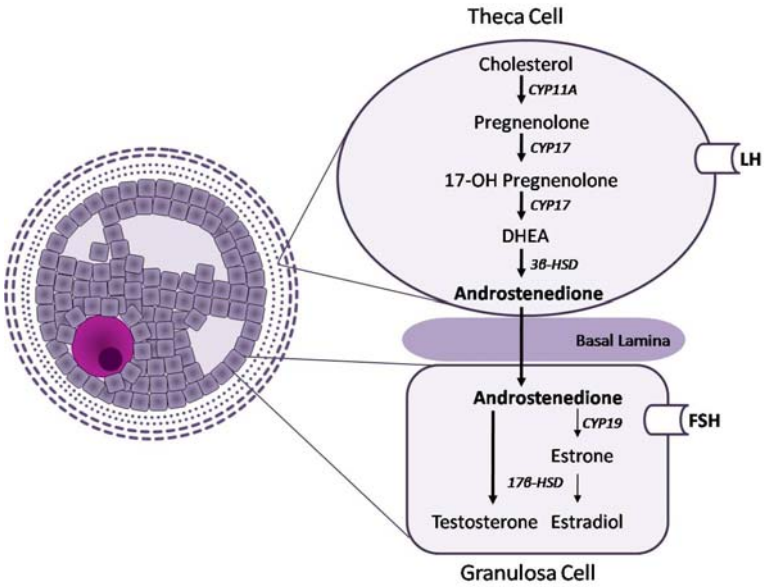

Figure 2. Ovarian steroidogenesis in theca and granulosa cells. LH stimulates theca cells to express the enzymes essential for the production of androstenedione (CYP11A, CYP17, and 3β-HSD). The androstenedione diffuses across the basal lamina into the granulosa cells where it is metabolized to estradiol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

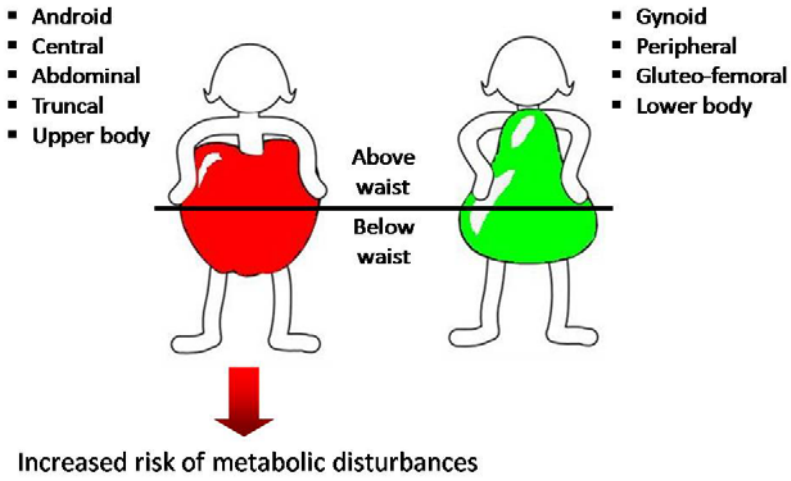

Figure 3. Abdominal, and in particular, visceral fat deposition (above waist, apple) is associated with metabolic disturbances such as insulin resistance, impaired glucose tolerance, dyslipidemia, hypertension, type 2 diabetes mellitus and cardiovascular disease, while accumulation of fat on hips, thighs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

TNFα 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]

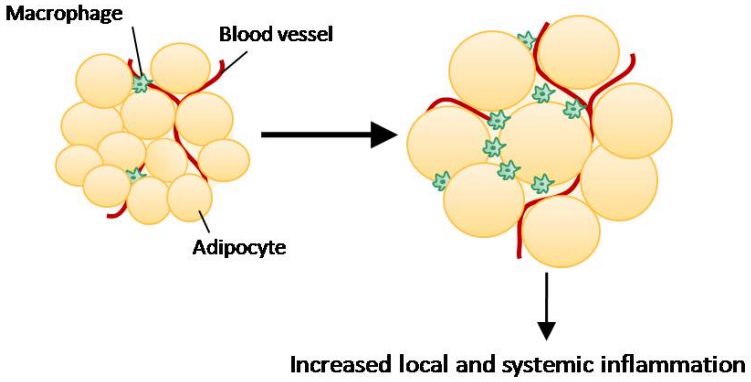

Figure 4. Adipose tissue expansion, especially hypertrophic growth, is associated with an increased infiltration of macrophages.[198] Many of the macrophages are aggregated around adipocytes, forming what are known as crown-like structures (CLS).

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)-1α.[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]

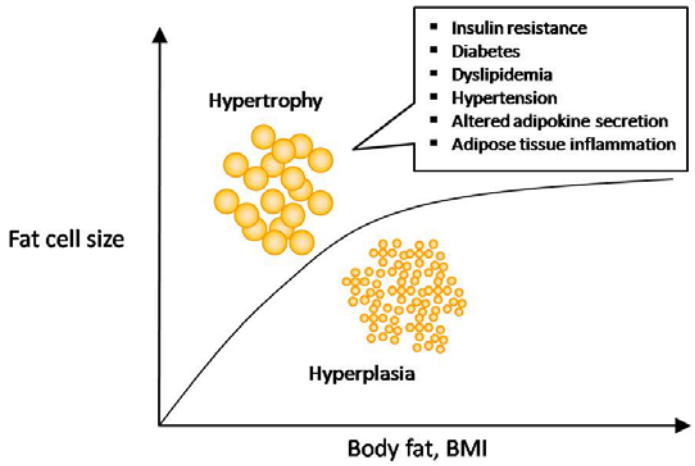

Figure 5. Adipose tissue expands by increasing the size of preexisting adipocytes (hypertrophy), by generating new small adipocytes (hyperplasia), or both. There is a large interindividual variation in mean fat cell size both in lean and obese subjects. Adipose tissue hypertrophy is associated with metabolic 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 (PPARγ) 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 infiltration[202] 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 neuroendrocrine 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 strengthen 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]

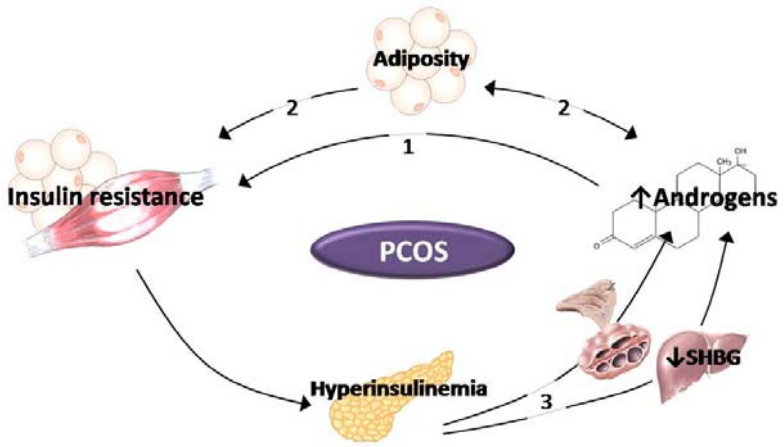

Figure 6. The hallmarks of PCOS – hyperinsulinemia and hyperandrogenism – are part of a vicious circle. 1) Androgens can directly influence insulin action in adipocytes and skeletal muscle. 2) Androgens can also influence insulin sensitivity indirectly via effects on lipid metabolism, adiposity/body 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 antiprogesterone 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 Aδ afferents and possibly unmyelinated C afferents.[318,319] The activation of Aδ 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]

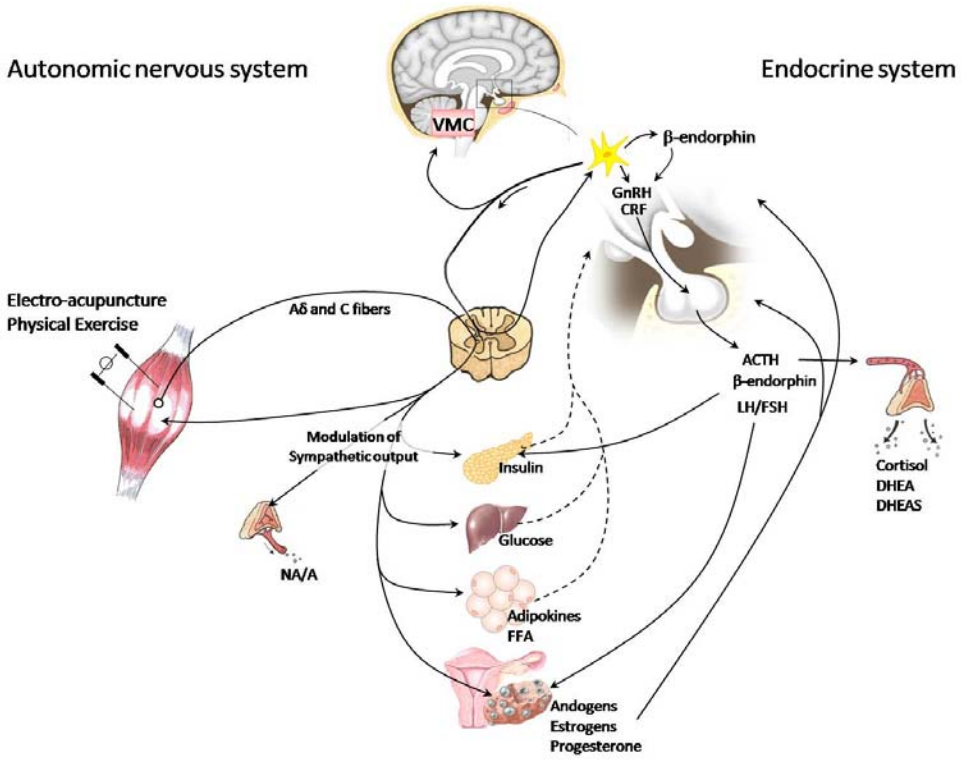

Figure 7. A hypothetical model of the effects of low-frequency EA and physical exercise in PCOS. Muscle contractions caused by low frequency EA and physical exercise excite ergoreceptors in the muscle which in turn activate afferent Aδ and C fibers. The signals are transmitted to the spinal cord (segmental 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 pancreas[334] 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 humans[343-347] and animals[348] 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.

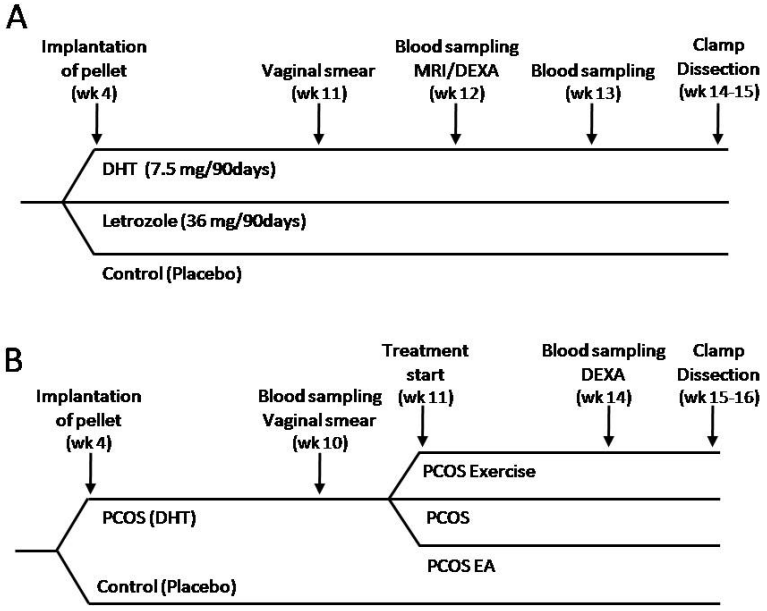

Figure 8. A) Study design and time axis of paper I. B) Study design and time axis of papers II and III. (DHT – dihydrotestosterone, wk – week of age, DEXA – dual-energy X-ray absorptiometry, MRI – magnetic resonance imaging)

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 (Aδ 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/m^2).

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/amenorrhea[6] – 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 height^2 (m^2)] 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 ($\pi d\ [3(S.D.)^2 + d^2)]/6$), 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 $2^{-\Delta\Delta CT}$.[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, $2^{-\Delta\Delta CT}$.

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 macrophages[198]) 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.

	Experimental studies		Human study
Analyte	Paper I	Paper II	Paper IV
Progesterone	IA		
Testosterone	IA		MS
17β-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 17β-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 17β-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, 17β-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 ± standard error of the mean (SEM) in papers I-III and as mean ± 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 *vs.* Controls	Exercise *vs.* untreated PCOS	EA *vs.* untreated PCOS
Lep	↑	↓	↓
Il6	↑	↓	=
Tnf	=	=	=
Pparg	=	=	↓
Ucp2	↓	=	↑
Adrb3	↑	=	↓
Ar	=	=	↓
Ngf	↑	↓	↓
Ngfr	=	=	=
Npy	↑	↓	↓

[A]Gene 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 17ß-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.

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

	In general [A]	Paper IV	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 administrating 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.

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REFERENCES

1. Ehrmann DA. Polycystic Ovary Syndrome. N Engl J Med 2005; 352:1223-1236
2. Jonard S, Dewailly D. The follicular excess in polycystic ovaries, due to intra-ovarian hyperandrogenism, may be the main culprit for the follicular arrest. Hum Reprod Update 2004; 10:107-117
3. Teede H, Hutchison S, Zoungas S, Meyer C. Insulin resistance, the metabolic syndrome, diabetes, and cardiovascular disease risk in women with PCOS. Endocrine 2006; 30:45-53
4. Stein I, Leventhal M. Amenorrhoea associated with bilateral polycystic ovaries. Am J Obstet Gynecol 1935; 29:181-191
5. Zawadzki J, Dunaif A. Diagnostic criteria for polycystic ovary syndrome: towards a rational approach. Boston: Blackwell; 1992
6. The Rotterdam ESHRE/ASRM sponsored PCOS consensus workshop group. Revised 2003 consensus on diagnostic criteria and long-term health risks related to polycystic ovary syndrome (PCOS). Hum Reprod 2004; 19:41-47
7. Azziz R, Carmina E, Dewailly D, Diamanti-Kandarakis E, Escobar-Morreale HF, Futterweit W, Janssen OE, Legro RS, Norman RJ, Taylor AE, Witchel SF. Criteria for Defining Polycystic Ovary Syndrome as a Predominantly Hyperandrogenic Syndrome: An Androgen Excess Society Guideline. J Clin Endocrinol Metab 2006; 91:4237-4245
8. Azziz R, Woods KS, Reyna R, Key TJ, Knochenhauer ES, Yildiz BO. The Prevalence and Features of the Polycystic Ovary Syndrome in an Unselected Population. J Clin Endocrinol Metab 2004; 89:2745-2749
9. March WA, Moore VM, Willson KJ, Phillips DI, Norman RJ, Davies MJ. The prevalence of polycystic ovary syndrome in a community sample assessed under contrasting diagnostic criteria. Hum Reprod 2009 Nov 12 [Epub ahead of print]
10. Schüring AN, Schulte N, Sonntag B, Kiesel L. Androgens and Insulin - Two Key Players in Polycystic Ovary Syndrome. Gynäkol Geburtshilfliche Rundsch 2008; 48:9-15
11. Marshall JC, Griffin ML. The role of changing pulse frequency in the regulation of ovulation. Hum Reprod 1993; 8:57-61
12. Taylor AE, McCourt B, Martin KA, Anderson EJ, Adams JM, Schoenfeld D, Hall JE. Determinants of Abnormal Gonadotropin Secretion in Clinically Defined Women with Polycystic Ovary Syndrome. J Clin Endocrinol Metab 1997; 82:2248-2256
13. Rebar R, Judd HL, Yen SS, Rakoff J, Vandenberg G, Naftolin F. Characterization of the inappropriate gonadotropin secretion in polycystic ovary syndrome. J Clin Invest 1976; 57:1320-1329
14. Blank SK, McCartney CR, Marshall JC. The origins and sequelae of abnormal neuroendocrine function in polycystic ovary syndrome. Hum Reprod Update 2006; 12:351-361
15. Hayes FJ, Taylor AE, Martin KA, Hall JE. Use of a Gonadotropin-Releasing Hormone Antagonist as a Physiologic Probe in Polycystic Ovary Syndrome: Assessment of Neuroendocrine and Androgen Dynamics. J Clin Endocrinol Metab 1998; 83:2343-2349

16. Pastor CL, Griffin-Korf ML, Aloi JA, Evans WS, Marshall JC. Polycystic Ovary Syndrome: Evidence for Reduced Sensitivity of the Gonadotropin-Releasing Hormone Pulse Generator to Inhibition by Estradiol and Progesterone. J Clin Endocrinol Metab 1998; 83:582-590
17. Hillier SG, Whitelaw PF, Smyth CD. Follicular oestrogen synthesis: the 'two-cell, two-gonadotrophin' model revisited. Mol Cell Endocrinol 1994; 100:51-54
18. Magoffin DA. Ovarian theca cell. Int J Biochem Cell Biol 2005; 37:1344-1349
19. Nelson VL, Legro RS, Strauss JF, III, McAllister JM. Augmented Androgen Production Is a Stable Steroidogenic Phenotype of Propagated Theca Cells from Polycystic Ovaries. Mol Endocrinol 1999; 13:946-957
20. Gilling-Smith C, Willis DS, Beard RW, Franks S. Hypersecretion of androstenedione by isolated thecal cells from polycystic ovaries. J Clin Endocrinol Metab 1994; 79:1158-1165
21. Nelson VL, Qin K-N, Rosenfield RL, Wood JR, Penning TM, Legro RS, Strauss JF, III, McAllister JM. The biochemical basis for increased testosterone production in theca cells propagated from patients with polycystic ovary syndrome. J Clin Endocrinol Metab 2001; 86:5925-5933
22. Wickenheisser JK, Quinn PG, Nelson VL, Legro RS, Strauss JF, III, McAllister. JM. Differential Activity of the Cytochrome P450 17α-Hydroxylase and Steroidogenic Acute Regulatory Protein Gene Promoters in Normal and Polycystic Ovary Syndrome Theca Cells. J Clin Endocrinol Metab 2000; 85:2304-2311
23. Yildiz BO, Azziz R. The adrenal and polycystic ovary syndrome. Rev Endocr Metab Disord 2007; 8:331-342
24. Azziz R, Black V, Hines GA, Fox LM, Boots LR. Adrenal Androgen Excess in the Polycystic Ovary Syndrome: Sensitivity and Responsivity of the Hypothalamic-Pituitary-Adrenal Axis. J Clin Endocrinol Metab 1998; 83:2317-2323
25. Tsilchorozidou T, Honour JW, Conway GS. Altered cortisol metabolism in polycystic ovary syndrome: insulin enhances 5α-reduction but not the elevated adrenal steroid production rates. J Clin Endocrinol Metab 2003; 88:5907-5913
26. Baillargeon J-P, Nestler JE. Polycystic Ovary Syndrome: A Syndrome of Ovarian Hypersensitivity to Insulin? J Clin Endocrinol Metab 2006; 91:22-24
27. Nestler JE. Insulin regulation of human ovarian androgens. Hum Reprod 1997; 12 Suppl 1:53-62
28. Poretsky L, Cataldo NA, Rosenwaks Z, Giudice LC. The Insulin-Related Ovarian Regulatory System in Health and Disease. Endocr Rev 1999; 20:535-582
29. Diamanti-Kandarakis E, Argyrakopoulou G, Economou F, Kandaraki E, Koutsilieris M. Defects in insulin signaling pathways in ovarian steroidogenesis and other tissues in polycystic ovary syndrome (PCOS). J Steroid Biochem Mol Biol 2008; 109:242-246
30. Duleba AJ, Spaczynski RZ, Olive DL. Insulin and insulin-like growth factor I stimulate the proliferation of human ovarian theca-interstitial cells. Fertil Steril 1998; 69:335-340
31. Poretsky L, Clemons J, Bogovich K. Hyperinsulinemia and human chorionic gonadotropin synergistically promote the growth of ovarian follicular cysts in rats. Metabolism 1992; 41:903-910
32. Nestler JE, Powers LP, Matt DW, Steingold KA, Plymate SR, Rittmaster RS, Clore JN, Blackard WG. A direct effect of hyperinsulinemia on serum sex hormone-binding

globulin levels in obese women with the polycystic ovary syndrome. J Clin Endocrinol Metab 1991; 72:83-89
33. Yki-Järvinen H, Mäkimattila S, Utriainen T, Rutanen EM. Portal insulin concentrations rather than insulin sensitivity regulate serum sex hormone-binding globulin and insulin-like growth factor binding protein 1 in vivo. J Clin Endocrinol Metab 1995; 80:3227-3232
34. Moghetti P, Castello R, Negri C, Tosi F, Spiazzi GG, Brun E, Balducci R, Toscano V, Muggeo M. Insulin infusion amplifies 17 alpha-hydroxycorticosteroid intermediates response to adrenocorticotropin in hyperandrogenic women: apparent relative impairment of 17,20-lyase activity. J Clin Endocrinol Metab 1996; 81:881-886
35. Adashi EY, Hsueh AJ, Yen SS. Insulin enhancement of luteinizing hormone and follicle-stimulating hormone release by cultured pituitary cells. Endocrinology 1981; 108:1441-1449
36. Moret M, Stettler R, Rodieux F, Gaillard RC, Waeber G, Wirthner D, Giusti V, Tappy L, Pralong FP. Insulin modulation of luteinizing hormone secretion in normal female volunteers and lean polycystic ovary syndrome patients. Neuroendocrinology 2009; 89:131-139
37. Mehta RV, Patel KS, Coffler MS, Dahan MH, Yoo RY, Archer JS, Malcom PJ, Chang RJ. Luteinizing Hormone Secretion Is Not Influenced by Insulin Infusion in Women with Polycystic Ovary Syndrome Despite Improved Insulin Sensitivity during Pioglitazone Treatment. J Clin Endocrinol Metab 2005; 90:2136-2141
38. Patel K, Coffler MS, Dahan MH, Yoo RY, Lawson MA, Malcom PJ, Chang RJ. Increased Luteinizing Hormone Secretion in Women with Polycystic Ovary Syndrome Is Unaltered by Prolonged Insulin Infusion. J Clin Endocrinol Metab 2003; 88:5456-5461
39. Nestler JE. Metformin for the Treatment of the Polycystic Ovary Syndrome. N Engl J Med 2008; 358:47-54
40. Greiner M, Paredes A, Araya V, Lara HE. Role of stress and sympathetic innervation in the development of polycystic ovary syndrome. Endocrine 2005; 28:319-324
41. Aguado LI. Role of the central and peripheral nervous system in the ovarian function. Microsc Res Tech 2002; 59:462-473
42. Grassi G, Dell'Oro R, Facchini A, Quarti Trevano F, Bolla GB, Mancia G. Effect of central and peripheral body fat distribution on sympathetic and baroreflex function in obese normotensives. J Hypertens 2004; 22:2363-2369
43. Reaven GM, Lithell H, Landsberg L. Hypertension and Associated Metabolic Abnormalities -- The Role of Insulin Resistance and the Sympathoadrenal System. N Engl J Med 1996; 334:374-382
44. Fagius J. Sympathetic nerve activity in metabolic control - some basic concepts. Acta Physiol Scand 2003; 177:337-343
45. Semenova, II. [Adrenergic innervation of ovaries in Stein-Leventhal syndrome]. Vestn Akad Med Nauk SSSR 1969; 24:58-62
46. Heider U, Pedal I, Spanel-Borowski K. Increase in nerve fibers and loss of mast cells in polycystic and postmenopausal ovaries. Fertil Steril 2001; 75:1141-1147
47. Garcia-Rudaz C, Armando I, Levin G, Escobar ME, Barontini M. Peripheral catecholamine alterations in adolescents with polycystic ovary syndrome. Clin Endocrinol 1998; 49:221-228

48. Dissen GA, Garcia-Rudaz C, Paredes A, Mayer C, Mayerhofer A, Ojeda SR. Excessive ovarian production of nerve growth factor facilitates development of cystic ovarian morphology in mice and is a feature of polycystic ovarian syndrome in humans. Endocrinology 2009; 150:2906-2914
49. Yildirir A, Aybar F, Kabakci G, Yarali H, Oto A. Heart rate variability in young women with polycystic ovary syndrome. Ann Noninvasive Electrocardiol 2006; 11:306-312
50. Tekin G, Tekin A, KilIçarslan EB, Haydardedeoglu B, KatIrcIbasI T, Koçum T, Erol T, Çölkesen Y, Sezgin AT, Müderrisoglu H. Altered autonomic neural control of the cardiovascular system in patients with polycystic ovary syndrome. Int J Cardiol 2008; 130:49-55
51. Giallauria F, Palomba S, Manguso F, Vitelli A, Maresca L, Tafuri D, Lombardi G, Colao A, Vigorito C, Orio F. Abnormal heart rate recovery after maximal cardiopulmonary exercise stress testing in young overweight women with polycystic ovary syndrome. Clin Endocrinol 2008; 68:88-93
52. Sverrisdottir YB, Mogren T, Kataoka J, Janson PO, Stener-Victorin E. Is polycystic ovary syndrome associated with high sympathetic nerve activity and size at birth? Am J Physiol Endocrinol Metab 2008; 294:E576-E581
53. Kahsar-Miller MD, Nixon C, Boots LR, Go RC, Azziz R. Prevalence of polycystic ovary syndrome (PCOS) in first-degree relatives of patients with PCOS. Fertil Steril 2001; 75:53-58
54. Azziz R, Kashar-Miller MD. Family history as a risk factor for the polycystic ovary syndrome. J Pediatr Endocrinol Metab 2000; 13 Suppl 5:1303-1306
55. Vink JM, Sadrzadeh S, Lambalk CB, Boomsma DI. Heritability of Polycystic Ovary Syndrome in a Dutch Twin-Family Study. J Clin Endocrinol Metab 2006; 91:2100-2104
56. Baillargeon JP, Carpentier A. Brothers of women with polycystic ovary syndrome are characterised by impaired glucose tolerance, reduced insulin sensitivity and related metabolic defects. Diabetologia 2007; 50:2424-2432
57. Cooper HE, Spellacy WN, Prem KA, Cohen WD. Hereditary factors in the Stein-Leventhal syndrome. Am J Obstet Gynecol 1968; 100:371-387
58. Legro RS, Bentley-Lewis R, Driscoll D, Wang SC, Dunaif A. Insulin Resistance in the Sisters of Women with Polycystic Ovary Syndrome: Association with Hyperandrogenemia Rather Than Menstrual Irregularity. J Clin Endocrinol Metab 2002; 87:2128-2133
59. Legro RS, Driscoll D, Strauss JF, 3rd, Fox J, Dunaif A. Evidence for a genetic basis for hyperandrogenemia in polycystic ovary syndrome. Proc Natl Acad Sci U S A 1998; 95:14956-14960
60. Escobar-Morreale HF, Luque-Ramirez M, San Millan JL. The Molecular-Genetic Basis of Functional Hyperandrogenism and the Polycystic Ovary Syndrome. Endocr Rev 2005; 26:251-282
61. Dewailly D, Robert Y, Helln I, Ardaens Y, Thomas-Desrousseaux P, Lemaltre L, Fossati P. Ovarian stromal hypertrophy in hyperandrogenic women. Clin Endocrinol 1994; 41:557-562
62. Hughesdon PE. Morphology and morphogenesis of the Stein-Leventhal ovary and of so-called "hyperthecosis". Obstet Gynecol Surv 1982; 37:59-77

63. Balen AH, Laven JSE, Tan S-L, Dewailly D. Ultrasound assessment of the polycystic ovary: international consensus definitions. Hum Reprod Update 2003; 9:505-514
64. Webber LJ, Stubbs S, Stark J, Trew GH, Margara R, Hardy K, Franks S. Formation and early development of follicles in the polycystic ovary. Lancet 2003; 362:1017-1021
65. Franks S, Stark J, Hardy K. Follicle dynamics and anovulation in polycystic ovary syndrome. Hum Reprod Update 2008; 14:367-378
66. Franks S, Mason H, Willis D. Follicular dynamics in the polycystic ovary syndrome. Mol Cell Endocrinol 2000; 163:49-52
67. Vendola KA, Zhou J, Adesanya OO, Weil SJ, Bondy CA. Androgens stimulate early stages of follicular growth in the primate ovary. J Clin Invest 1998; 101:2622-2629
68. Nisenblat V, Norman RJ. Androgens and polycystic ovary syndrome. Curr Opin Endocrinol Diabetes Obes 2009; 16:224-231
69. Teixeira Filho FL, Baracat EC, Lee TH, Suh CS, Matsui M, Chang RJ, Shimasaki S, Erickson GF. Aberrant Expression of Growth Differentiation Factor-9 in Oocytes of Women with Polycystic Ovary Syndrome. J Clin Endocrinol Metab 2002; 87:1337-1344
70. Pigny P, Merlen E, Robert Y, Cortet-Rudelli C, Decanter C, Jonard S, Dewailly D. Elevated serum level of anti-mullerian hormone in patients with polycystic ovary syndrome: relationship to the ovarian follicle excess and to the follicular arrest. J Clin Endocrinol Metab 2003; 88:5957-5962
71. Willis D, Mason H, Gilling-Smith C, Franks S. Modulation by insulin of follicle-stimulating hormone and luteinizing hormone actions in human granulosa cells of normal and polycystic ovaries. J Clin Endocrinol Metab 1996; 81:302-309
72. Ehrmann DA, Liljenquist DR, Kasza K, Azziz R, Legro RS, Ghazzi MN. Prevalence and Predictors of the Metabolic Syndrome in Women with Polycystic Ovary Syndrome. J Clin Endocrinol Metab 2006; 91:48-53
73. Apridonidze T, Essah PA, Iuorno MJ, Nestler JE. Prevalence and Characteristics of the Metabolic Syndrome in Women with Polycystic Ovary Syndrome. J Clin Endocrinol Metab 2005; 90:1929-1935
74. Essah PA, Nestler JE. The metabolic syndrome in polycystic ovary syndrome. J Endocrinol Invest 2006; 29:270-280
75. Cussons AJ, Watts GF, Burke V, Shaw JE, Zimmet PZ, Stuckey BG. Cardiometabolic risk in polycystic ovary syndrome: a comparison of different approaches to defining the metabolic syndrome. Hum Reprod 2008; 23:2352-2358
76. Legro RS, Kunselman AR, Dodson WC, Dunaif A. Prevalence and Predictors of Risk for Type 2 Diabetes Mellitus and Impaired Glucose Tolerance in Polycystic Ovary Syndrome: A Prospective, Controlled Study in 254 Affected Women. J Clin Endocrinol Metab 1999; 84:165-169
77. Ehrmann DA, Barnes RB, Rosenfield RL, Cavaghan MK, Imperial J. Prevalence of impaired glucose tolerance and diabetes in women with polycystic ovary syndrome. Diabetes Care 1999; 22:141-146
78. Kauffman RP, Baker TE, Baker VM, DiMarino P, Castracane VD. Endocrine and metabolic differences among phenotypic expressions of polycystic ovary syndrome according to the 2003 Rotterdam consensus criteria. Am J Obstet Gynecol 2008; 198:670.e671-670.e610

79. Corbould A. Effects of androgens on insulin action in women: is androgen excess a component of female metabolic syndrome? Diabetes Metab Res Rev 2008; 24:520-532
80. DeFronzo RA, Jacot E, Jequier E, Maeder E, Wahren J, Felber JP. The effect of insulin on the disposal of intravenous glucose. Results from indirect calorimetry and hepatic and femoral venous catheterization. Diabetes 1981; 30:1000-1007
81. Minokoshi Y, Kahn CR, Kahn BB. Tissue-specific Ablation of the GLUT4 Glucose Transporter or the Insulin Receptor Challenges Assumptions about Insulin Action and Glucose Homeostasis. J Biol Chem 2003; 278:33609-33612
82. O'Meara N, Blackman J, Ehrmann D, Barnes R, Jaspan J, Rosenfield R, Polonsky K. Defects in β-cell function in functional ovarian hyperandrogenism. J Clin Endocrinol Metab 1993; 76:1241-1247
83. Ehrmann DA, Sturis J, Byrne MM, Karrison T, Rosenfield RL, Polonsky KS. Insulin secretory defects in polycystic ovary syndrome. Relationship to insulin sensitivity and family history of non-insulin-dependent diabetes mellitus. J Clin Invest 1995; 96:520-527
84. Ciampelli M, Fulghesu A, Cucinelli F, Pavone V, Caruso A, Mancuso S, Lanzone A. Heterogeneity in β cell activity, hepatic insulin clearance and peripheral insulin sensitivity in women with polycystic ovary syndrome. Hum Reprod 1997; 12:1897-1901
85. Archard C, Thiers J. Le virilisme pilaire et son association a l'insuffisance glycolytique (diabete des femmes a barbe). Bull Acad Natl Med 1921; 86:51
86. Burghen GA, Givens JR, Kitabchi AE. Correlation of hyperandrogenism with hyperinsulinism in polycystic ovarian disease. J Clin Endocrinol Metab 1980; 50:113-116
87. Dunaif A, Segal KR, Futterweit W, Dobrjansky A. Profound peripheral insulin resistance, independent of obesity, in polycystic ovary syndrome. Diabetes 1989; 38:1165-1174
88. Morales AJ, Laughlin GA, Butzow T, Maheshwari H, Baumann G, Yen SS. Insulin, somatotropic, and luteinizing hormone axes in lean and obese women with polycystic ovary syndrome: common and distinct features. J Clin Endocrinol Metab 1996; 81:2854-2864
89. Carmina E, Koyama T, Chang L, Stanczyk FZ, Lobo RA. Does ethnicity influence the prevalence of adrenal hyperandrogenism and insulin resistance in polycystic ovary syndrome? Am J Obstet Gynecol 1992; 167:1807-1812
90. Carmina E, Lobo RA. Use of fasting blood to assess the prevalence of insulin resistance in women with polycystic ovary syndrome. Fertil Steril 2004; 82:661-665
91. Morin-Papunen LC, Vauhkonen I, Koivunen RM, Ruokonen A, Tapanainen JS. Insulin sensitivity, insulin secretion, and metabolic and hormonal parameters in healthy women and women with polycystic ovarian syndrome. Hum Reprod 2000; 15:1266-1274
92. Holte J, Gennarelli G, Berne C, Bergh T, Lithell H. Elevated ambulatory day-time blood pressure in women with polycystic ovary syndrome: a sign of a pre-hypertensive state? Hum Reprod 1996; 11:23-28
93. Carmina E, Bucchieri S, Esposito A, Del Puente A, Mansueto P, Orio F, Di Fede G, Rini G. Abdominal Fat Quantity and Distribution in Women with Polycystic Ovary

Syndrome and Extent of Its Relation to Insulin Resistance. J Clin Endocrinol Metab 2007; 92:2500-2505
94. Puder JJ, Varga S, Kraenzlin M, De Geyter C, Keller U, Muller B. Central Fat Excess in Polycystic Ovary Syndrome: Relation to Low-Grade Inflammation and Insulin Resistance. J Clin Endocrinol Metab 2005; 90:6014-6021
95. Ek I, Arner P, Ryden M, Holm C, Thorne A, Hoffstedt J, Wahrenberg H. A Unique Defect in the Regulation of Visceral Fat Cell Lipolysis in the Polycystic Ovary Syndrome as an Early Link to Insulin Resistance. Diabetes 2002; 51:484-492
96. Diamanti-Kandarakis E, Papavassiliou AG. Molecular mechanisms of insulin resistance in polycystic ovary syndrome. Trends Mol Med 2006; 12:324-332
97. Dunaif A. Insulin Resistance and the Polycystic Ovary Syndrome: Mechanism and Implications for Pathogenesis. Endocr Rev 1997; 18:774-800
98. Corbould A. Chronic testosterone treatment induces selective insulin resistance in subcutaneous adipocytes of women. J Endocrinol 2007; 192:585-594
99. Allemand MC, Irving BA, Asmann YW, Klaus KA, Tatpati L, Coddington CC, Nair KS. Effect of Testosterone on Insulin Stimulated IRS1 Ser Phosphorylation in Primary Rat Myotubes - A Potential Model for PCOS-Related Insulin Resistance. PLoS ONE 2009; 4:e4274
100. Corbould A, Kim Y-B, Youngren JF, Pender C, Kahn BB, Lee A, Dunaif A. Insulin resistance in the skeletal muscle of women with PCOS involves intrinsic and acquired defects in insulin signaling. Am J Physiol Endocrinol Metab 2005; 288:E1047-1054
101. Holmäng A, Larsson BM, Brzezinska Z, Björntorp P. Effects of short-term testosterone exposure on insulin sensitivity of muscles in female rats. Am J Physiol Endocrinol Metab 1992; 262:E851-855
102. Holmäng A, Svedberg J, Jennische E, Björntorp P. Effects of testosterone on muscle insulin sensitivity and morphology in female rats. Am J Physiol Endocrinol Metab 1990; 259:E555-E560
103. Holmäng A, Niklasson M, Rippe B, Lönnroth P. Insulin insensitivity and delayed transcapillary delivery of insulin in oophorectomized rats treated with testosterone. Acta Physiol Scand 2001; 171:427-438
104. Rincon J, Holmäng A, Wahlström EO, Lönnroth P, Björntorp P, Zierath JR, Wallberg-Henriksson H. Mechanisms behind insulin resistance in rat skeletal muscle after oophorectomy and additional testosterone treatment. Diabetes 1996; 45:615-621
105. Ciaraldi TP, Aroda V, Mudaliar S, Chang RJ, Henry RR. Polycystic Ovary Syndrome Is Associated with Tissue-Specific Differences in Insulin Resistance. J Clin Endocrinol Metab 2009; 94:157-163
106. Kopelman PG. Obesity as a medical problem. Nature 2000; 404:635-643
107. Ruderman N, Chisholm D, Pi-Sunyer X, Schneider S. The metabolically obese, normal-weight individual revisited. Diabetes 1998; 47:699-713
108. Brochu M, Tchernof A, Dionne IJ, Sites CK, Eltabbakh GH, Sims EAH, Poehlman ET. What Are the Physical Characteristics Associated with a Normal Metabolic Profile Despite a High Level of Obesity in Postmenopausal Women? J Clin Endocrinol Metab 2001; 86:1020-1025
109. Björntorp P. The regulation of adipose tissue distribution in humans. Int J Obes Relat Metab Disord 1996; 20:291-302

110. Ross R, Shaw K, Rissanen J, Martel Y, de Guise J, Avruch L. Sex differences in lean and adipose tissue distribution by magnetic resonance imaging: anthropometric relationships. Am J Clin Nutr 1994; 59:1277-1285
111. Lemieux S, Prud'homme D, Bouchard C, Tremblay A, Despres J. Sex differences in the relation of visceral adipose tissue accumulation to total body fatness. Am J Clin Nutr 1993; 58:463-467
112. Vague J. The degree of masculine differentiation of obesities: a factor determining predisposition to diabetes, atherosclerosis, gout, and uric calculous disease. Am J Clin Nutr 1956; 4:20-34
113. Pi-Sunyer FX. The Epidemiology of Central Fat Distribution in Relation to Disease. Nutr Rev 2004; 62:S120-S126
114. Ohlson LO, Larsson B, Svardsudd K, Welin L, Eriksson H, Wilhelmsen L, Bjorntorp P, Tibblin G. The influence of body fat distribution on the incidence of diabetes mellitus. 13.5 years of follow-up of the participants in the study of men born in 1913. Diabetes 1985; 34:1055-1058
115. Lundgren H, Bengtsson C, Blohme G, Lapidus L, Sjostrom L. Adiposity and adipose tissue distribution in relation to incidence of diabetes in women: results from a prospective population study in Gothenburg, Sweden. Int J Obes 1989; 13:413-423
116. Canoy D, Luben R, Welch A, Bingham S, Wareham N, Day N, Khaw K-T. Fat distribution, body mass index and blood pressure in 22 090 men and women in the Norfolk cohort of the European Prospective Investigation into Cancer and Nutrition (EPIC-Norfolk) study. J Hypertens 2004; 22:2067-2074
117. Panagiotakos D, Pitsavos C, Skoumas Y, Lentzas Y, Papadimitriou L, Chrysohoou C, Stefanadis C. Abdominal obesity, blood glucose and apolipoprotein B levels are the best predictors of the incidence of hypercholesterolemia (2001-2006) among healthy adults: the ATTICA Study. Lipids Health Dis 2008; 7:11
118. Krotkiewski M, Björntorp P, Sjöström L, Smith U. Impact of obesity on metabolism in men and women. Importance of regional adipose tissue distribution. J Clin Invest 1983; 72:1150-1162
119. Evans DJ, Hoffmann RG, Kalkhoff RK, Kissebah AH. Relationship of body fat topography to insulin sensitivity and metabolic profiles in premenopausal women. Metabolism 1984; 33:68-75
120. Bengtsson C, Björkelund C, Lapidus L, Lissner L. Associations of serum lipid concentrations and obesity with mortality in women: 20 year follow up of participants in prospective population study in Gothenburg, Sweden. BMJ 1993; 307:1385-1388
121. Mathieu P, Poirier P, Pibarot P, Lemieux I, Despres J-P. Visceral Obesity: The Link Among Inflammation, Hypertension, and Cardiovascular Disease. Hypertension 2009; 53:577-584
122. Abate N, Garg A. Heterogeneity in adipose tissue metabolism: causes, implications and management of regional adiposity. Prog Lipid Res 1995; 34:53-70
123. Ross R, Aru J, Freeman J, Hudson R, Janssen I. Abdominal adiposity and insulin resistance in obese men. Am J Physiol Endocrinol Metab 2002; 282:E657-663
124. Ross R, Freeman J, Hudson R, Janssen I. Abdominal Obesity, Muscle Composition, and Insulin Resistance in Premenopausal Women. J Clin Endocrinol Metab 2002; 87:5044-5051

125. Carr DB, Utzschneider KM, Hull RL, Kodama K, Retzlaff BM, Brunzell JD, Shofer JB, Fish BE, Knopp RH, Kahn SE. Intra-abdominal fat is a major determinant of the National Cholesterol Education Program Adult Treatment Panel III criteria for the metabolic syndrome. Diabetes 2004; 53:2087-2094
126. Lord J, Thomas R, Fox B, Acharya U, Wilkin T. The central issue? Visceral fat mass is a good marker of insulin resistance and metabolic disturbance in women with polycystic ovary syndrome. BJOG 2006; 113:1203-1209
127. Piché M-É, Weisnagel SJ, Corneau L, Nadeau A, Bergeron J, Lemieux S. Contribution of Abdominal Visceral Obesity and Insulin Resistance to the Cardiovascular Risk Profile of Postmenopausal Women. Diabetes 2005; 54:770-777
128. Garg A. Regional adiposity and insulin resistance. J Clin Endocrinol Metab 2004; 89:4206-4210
129. Pasquali R, Gambineri A, Pagotto U. The impact of obesity on reproduction in women with polycystic ovary syndrome. BJOG 2006; 113:1148-1159
130. Björntorp P. Hyperandrogenicity in women - a prediabetic condition? J Intern Med 1993; 234:579-583
131. Lovejoy JC, Bray GA, Bourgeois MO, Macchiavelli R, Rood JC, Greeson C, Partington C. Exogenous androgens influence body composition and regional body fat distribution in obese postmenopausal women - a clinical research center study. J Clin Endocrinol Metab 1996; 81:2198-2203
132. Kirchengast S, Huber J. Body composition characteristics and body fat distribution in lean women with polycystic ovary syndrome. Hum Reprod 2001; 16:1255-1260
133. Yildirim B, Sabir N, Kaleli B. Relation of intra-abdominal fat distribution to metabolic disorders in nonobese patients with polycystic ovary syndrome. Fertil Steril 2003; 79:1358-1364
134. Yucel A, Noyan V, Sagsoz N. The association of serum androgens and insulin resistance with fat distribution in polycystic ovary syndrome. Eur J Obstet Gynecol Reprod Biol 2006; 126:81-86
135. Horejsi R, Möller R, Rackl S, Giuliani A, Freytag U, Crailsheim K, Sudi K, Tafeit E. Android subcutaneous adipose tissue topography in lean and obese women suffering from PCOS: Comparison with type 2 diabetic women. Am J Phys Anthropol 2004; 124:275-281
136. Cascella T, Palomba S, De Sio I, Manguso F, Giallauria F, De Simone B, Tafuri D, Lombardi G, Colao A, Orio F. Visceral fat is associated with cardiovascular risk in women with polycystic ovary syndrome. Hum Reprod 2008; 23:153-159
137. Barber TM, Golding SJ, Alvey C, Wass JAH, Karpe F, Franks S, McCarthy MI. Global Adiposity Rather Than Abnormal Regional Fat Distribution Characterizes Women with Polycystic Ovary Syndrome. J Clin Endocrinol Metab 2008; 93:999-1004
138. Tchernof A, Despres JP. Sex steroid hormones, sex hormone-binding globulin, and obesity in men and women. Horm Metab Res 2000; 32:526-536
139. Kiddy DS, Sharp PS, White DM, Scanlon MF, Mason HD, Bray CS, Polson DW, Reed MJ, Franks S. Differences in clinical and endocrine features between obese and non-obese subjects with polycystic ovary syndrome: an analysis of 263 consecutive cases. Clin Endocrinol (Oxf) 1990; 32:213-220

140. Holte J, Bergh T, Gennarelli G, Wide L. The independent effects of polycystic ovary syndrome and obesity on serum concentrations of gonadotrophins and sex steroids in premenopausal women. Clin Endocrinol (Oxf) 1994; 41:473-481
141. Evans DJ, Barth JH, Burke CW. Body fat topography in women with androgen excess. Int J Obes 1988; 12:157-162
142. Kirschner MA, Samojlik E, Drejka M, Szmal E, Schneider G, Ertel N. Androgen-estrogen metabolism in women with upper body versus lower body obesity. J Clin Endocrinol Metab 1990; 70:473-479
143. Hartz AJ, Barboriak PN, Wong A, Katayama KP, Rimm AA. The association of obesity with infertility and related menstural abnormalities in women. Int J Obes 1979; 3:57-73
144. Rich-Edwards JW, Spiegelman D, Garland M, Hertzmark E, Hunter DJ, Colditz GA, Willett WC, Wand H, Manson JE. Physical activity, body mass index, and ovulatory disorder infertility. Epidemiology 2002; 13:184-190
145. Galtier-Dereure F, Pujol P, Dewailly D, Bringer J. Choice of stimulation in polycystic ovarian syndrome: the influence of obesity. Hum Reprod 1997; 12 Suppl 1:88-96
146. Balen A, Platteau P, Andersen A, Devroey P, Sørensen P, Helmgaard L, Arce J-C. The influence of body weight on response to ovulation induction with gonadotrophins in 335 women with World Health Organization group II anovulatory infertility. BJOG 2006; 113:1195-1202
147. Legro RS, Barnhart HX, Schlaff WD, Carr BR, Diamond MP, Carson SA, Steinkampf MP, Coutifaris C, McGovern PG, Cataldo NA, Gosman GG, Nestler JE, Giudice LC, Leppert PC, Myers ER. Clomiphene, Metformin, or Both for Infertility in the Polycystic Ovary Syndrome. N Engl J Med 2007; 356:551-566
148. Hirschberg AL. Polycystic ovary syndrome, obesity and reproductive implications. Womens Health 2009; 5:529-542
149. Kiddy DS, Hamilton-Fairley D, Bush A, Short F, Anyaoku V, Reed MJ, Franks S. Improvement in endocrine and ovarian function during dietary treatment of obese women with polycystic ovary syndrome. Clin Endocrinol (Oxf) 1992; 36:105-111
150. Crosignani PG, Colombo M, Vegetti W, Somigliana E, Gessati A, Ragni G. Overweight and obese anovulatory patients with polycystic ovaries: parallel improvements in anthropometric indices, ovarian physiology and fertility rate induced by diet. Hum Reprod 2003; 18:1928-1932
151. Huber-Buchholz MM, Carey DGP, Norman RJ. Restoration of reproductive potential by lifestyle modification in obese polycystic ovary syndrome: role of insulin sensitivity and luteinizing hormone. J Clin Endocrinol Metab 1999; 84:1470-1474
152. Holte J, Bergh T, Berne C, Wide L, Lithell H. Restored insulin sensitivity but persistently increased early insulin secretion after weight loss in obese women with polycystic ovary syndrome. J Clin Endocrinol Metab 1995; 80:2586-2593
153. Björntorp P. "Portal" adipose tissue as a generator of risk factors for cardiovascular disease and diabetes. Arteriosclerosis 1990; 10:493-496
154. Hoffstedt J, Arner P, Hellers G, Lonnqvist F. Variation in adrenergic regulation of lipolysis between omental and subcutaneous adipocytes from obese and non-obese men. J Lipid Res 1997; 38:795-804
155. Arner P. Differences in lipolysis between human subcutaneous and omental adipose tissues. Ann Med 1995; 27:435-438

156. Kotronen A, Juurinen L, Tiikkainen M, Vehkavaara S, Yki-Järvinen H. Increased Liver Fat, Impaired Insulin Clearance, and Hepatic and Adipose Tissue Insulin Resistance in Type 2 Diabetes. Gastroenterology 2008; 135:122-130
157. Cote M, Mauriege P, Bergeron J, Almeras N, Tremblay A, Lemieux I, Despres J-P. Adiponectinemia in Visceral Obesity: Impact on Glucose Tolerance and Plasma Lipoprotein and Lipid Levels in Men. J Clin Endocrinol Metab 2005; 90:1434-1439
158. Tsigos C, Kyrou I, Chala E, Tsapogas P, Stavridis JC, Raptis SA, Katsilambros N. Circulating tumor necrosis factor alpha concentrations are higher in abdominal versus peripheral obesity. Metabolism 1999; 48:1332-1335
159. Berg AH, Scherer PE. Adipose Tissue, Inflammation, and Cardiovascular Disease. Circ Res 2005; 96:939-949
160. Fried SK, Bunkin DA, Greenberg AS. Omental and Subcutaneous Adipose Tissues of Obese Subjects Release Interleukin-6: Depot Difference and Regulation by Glucocorticoid. J Clin Endocrinol Metab 1998; 83:847-850
161. Liu A, McLaughlin T, Liu T, Sherman A, Yee G, Abbasi F, Lamendola C, Morton J, Cushman SW, Reaven GM, Tsao PS. Differential Intra-abdominal Adipose Tissue Profiling in Obese, Insulin-resistant Women. Obes Surg 2009; 19:1564-1573
162. Despres J-P, Lemieux I. Abdominal obesity and metabolic syndrome. Nature 2006; 444:881-887
163. Schaffer JE. Lipotoxicity: when tissues overeat. Curr Opin Lipidol 2003; 14:281-287
164. Ravussin E, Smith SR. Increased Fat Intake, Impaired Fat Oxidation, and Failure of Fat Cell Proliferation Result in Ectopic Fat Storage, Insulin Resistance, and Type 2 Diabetes Mellitus. Ann N Y Acad Sci 2002; 967:363-378
165. Greco AV, Mingrone G, Giancaterini A, Manco M, Morroni M, Cinti S, Granzotto M, Vettor R, Camastra S, Ferrannini E. Insulin Resistance in Morbid Obesity. Diabetes 2002; 51:144-151
166. Krssak M, Falk Petersen K, Dresner A, DiPietro L, Vogel SM, Rothman DL, Shulman GI, Roden M. Intramyocellular lipid concentrations are correlated with insulin sensitivity in humans: a 1H NMR spectroscopy study. Diabetologia 1999; 42:113-116
167. Koyama K, Chen G, Lee Y, Unger RH. Tissue triglycerides, insulin resistance, and insulin production: implications for hyperinsulinemia of obesity. Am J Physiol Endocrinol Metab 1997; 273:E708-713
168. Yu C, Chen Y, Cline GW, Zhang D, Zong H, Wang Y, Bergeron R, Kim JK, Cushman SW, Cooney GJ, Atcheson B, White MF, Kraegen EW, Shulman GI. Mechanism by Which Fatty Acids Inhibit Insulin Activation of Insulin Receptor Substrate-1 (IRS-1)-associated Phosphatidylinositol 3-Kinase Activity in Muscle. J Biol Chem 2002; 277:50230-50236
169. Hauner H. Secretory factors from human adipose tissue and their functional role. Proc Nutr Soc 2005; 64:163-169
170. Trayhurn P, Wood IS. Adipokines: inflammation and the pleiotropic role of white adipose tissue. Br J Nutr 2004; 92:347-355
171. Zhang Y, Proenca R, Maffei M, Barone M, Leopold L, Friedman JM. Positional cloning of the mouse obese gene and its human homologue. Nature 1994; 372:425-432
172. Fain JN, Madan AK, Hiler ML, Cheema P, Bahouth SW. Comparison of the Release of Adipokines by Adipose Tissue, Adipose Tissue Matrix, and Adipocytes from Visceral

and Subcutaneous Abdominal Adipose Tissues of Obese Humans. Endocrinology 2004; 145:2273-2282
173. Vidal H. Gene expression in visceral and subcutaneous adipose tissues. Ann Med 2001; 33:547-555
174. Matsubara M, Maruoka S, Katayose S. Inverse relationship between plasma adiponectin and leptin concentrations in normal-weight and obese women. Eur J Endocrinol 2002; 147:173-180
175. Mitchell M, Armstrong DT, Robker RL, Norman RJ. Adipokines: implications for female fertility and obesity. Reproduction 2005; 130:583-597
176. Moschos S, Chan JL, Mantzoros CS. Leptin and reproduction: a review. Fertil Steril 2002; 77:433-444
177. Goumenou AG, Matalliotakis IM, Koumantakis GE, Panidis DK. The role of leptin in fertility. Eur J Obstet Gynecol Reprod Biol 2003; 106:118-124
178. Mantzoros CS, Dunaif A, Flier JS. Leptin concentrations in the polycystic ovary syndrome. J Clin Endocrinol Metab 1997; 82:1687-1691
179. Laughlin GA, Morales AJ, Yen SSC. Serum leptin levels in women with polycystic ovary syndrome: the role of insulin resistance/hyperinsulinemia. J Clin Endocrinol Metab 1997; 82:1692-1696
180. Rouru J, Anttila L, Koskinen P, Penttila TA, Irjala K, Huupponen R, Koulu M. Serum leptin concentrations in women with polycystic ovary syndrome. J Clin Endocrinol Metab 1997; 82:1697-1700
181. Nishida M, Funahashi T, Shimomura I. Pathophysiological significance of adiponectin. Med Mol Morphol 2007; 40:55-67
182. Weyer C, Funahashi T, Tanaka S, Hotta K, Matsuzawa Y, Pratley RE, Tataranni PA. Hypoadiponectinemia in Obesity and Type 2 Diabetes: Close Association with Insulin Resistance and Hyperinsulinemia. J Clin Endocrinol Metab 2001; 86:1930-1935
183. Toulis KA, Goulis DG, Farmakiotis D, Georgopoulos NA, Katsikis I, Tarlatzis BC, Papadimas I, Panidis D. Adiponectin levels in women with polycystic ovary syndrome: a systematic review and a meta-analysis. Hum Reprod Update 2009; 15:297-307
184. Carmina E, Chu MC, Moran C, Tortoriello D, Vardhana P, Tena G, Preciado R, Lobo R. Subcutaneous and omental fat expression of adiponectin and leptin in women with polycystic ovary syndrome. Fertil Steril 2008; 89:642-648
185. Taponen S, Martikainen H, Jarvelin M-R, Laitinen J, Pouta A, Hartikainen A-L, Sovio U, McCarthy MI, Franks S, Ruokonen A. Hormonal Profile of Women with Self-Reported Symptoms of Oligomenorrhea and/or Hirsutism: Northern Finland Birth Cohort 1966 Study. J Clin Endocrinol Metab 2003; 88:141-147
186. Belanger C, Luu-The V, Dupont P, Tchernof A. Adipose tissue intracrinology: potential importance of local androgen/estrogen metabolism in the regulation of adiposity. Horm Metab Res 2002; 34:737-745
187. Quinkler M, Sinha B, Tomlinson JW, Bujalska IJ, Stewart PM, Arlt W. Androgen generation in adipose tissue in women with simple obesity - a site-specific role for 17β-hydroxysteroid dehydrogenase type 5. J Endocrinol 2004; 183:331-342
188. Deslypere JP, Verdonck L, Vermeulen A. Fat tissue: a steroid reservoir and site of steroid metabolism. J Clin Endocrinol Metab 1985; 61:564-570

189. Wellen KE, Hotamisligil GS. Obesity-induced inflammatory changes in adipose tissue. J Clin Invest 2003; 112:1785-1788
190. Forouhi NG, Sattar N, McKeigue PM. Relation of C-reactive protein to body fat distribution and features of the metabolic syndrome in Europeans and South Asians. Int J Obes Relat Metab Disord 2001; 25:1327-1331
191. Hotamisligil GS, Shargill NS, Spiegelman BM. Adipose expression of tumor necrosis factor-α: direct role in obesity-linked insulin resistance. Science 1993; 259:87-91
192. Poitou C, Viguerie N, Cancello R, Matteis R, Cinti S, Stich V, Coussieu C, Gauthier E, Courtine M, Zucker J, Barsh G, Saris W, Bruneval P, Basdevant A, Langin D, Clément K. Serum amyloid A: production by human white adipocyte and regulation by obesity and nutrition. Diabetologia 2005; 48:519-528
193. Sjöholm K, Palming J, Olofsson LE, Gummesson A, Svensson P-A, Lystig TC, Jennische E, Brandberg J, Torgerson JS, Carlsson B, Carlsson LM. A Microarray Search for Genes Predominantly Expressed in Human Omental Adipocytes: Adipose Tissue as a Major Production Site of Serum Amyloid A. J Clin Endocrinol Metab 2005; 90:2233-2239
194. Mohamed-Ali V, Goodrick S, Rawesh A, Katz DR, Miles JM, Yudkin JS, Klein S, Coppack SW. Subcutaneous Adipose Tissue Releases Interleukin-6, But Not Tumor Necrosis Factor-α, in Vivo. J Clin Endocrinol Metab 1997; 82:4196-4200
195. Visser M, Bouter LM, McQuillan GM, Wener MH, Harris TB. Elevated C-reactive protein levels in overweight and obese adults. JAMA 1999; 282:2131-2135
196. Pannacciulli N, Cantatore FP, Minenna A, Bellacicco M, Giorgino R, De Pergola G. C-reactive protein is independently associated with total body fat, central fat, and insulin resistance in adult women. Int J Obes Relat Metab Disord 2001; 25:1416-1420
197. Yudkin JS, Stehouwer CDA, Emeis JJ, Coppack SW. C-Reactive Protein in Healthy Subjects: Associations With Obesity, Insulin Resistance, and Endothelial Dysfunction: A Potential Role for Cytokines Originating From Adipose Tissue? Arterioscler Thromb Vasc Biol 1999; 19:972-978
198. Cinti S, Mitchell G, Barbatelli G, Murano I, Ceresi E, Faloia E, Wang S, Fortier M, Greenberg AS, Obin MS. Adipocyte death defines macrophage localization and function in adipose tissue of obese mice and humans. J Lipid Res 2005; 46:2347-2355
199. Weisberg SP, McCann D, Desai M, Rosenbaum M, Leibel RL, Ferrante AW, Jr. Obesity is associated with macrophage accumulation in adipose tissue. J Clin Invest 2003; 112:1796-1808
200. Xu H, Barnes GT, Yang Q, Tan G, Yang D, Chou CJ, Sole J, Nichols A, Ross JS, Tartaglia LA, Chen H. Chronic inflammation in fat plays a crucial role in the development of obesity-related insulin resistance. J Clin Invest 2003; 112:1821-1830
201. Kanda H, Tateya S, Tamori Y, Kotani K, Hiasa K, Kitazawa R, Kitazawa S, Miyachi H, Maeda S, Egashira K, Kasuga M. MCP-1 contributes to macrophage infiltration into adipose tissue, insulin resistance, and hepatic steatosis in obesity. J Clin Invest 2006; 116:1494-1505
202. Cancello R, Henegar C, Viguerie N, Taleb S, Poitou C, Rouault C, Coupaye M, Pelloux V, Hugol D, Bouillot JL, Bouloumie A, Barbatelli G, Cinti S, Svensson PA, Barsh GS, Zucker JD, Basdevant A, Langin D, Clement K. Reduction of macrophage infiltration

and chemoattractant gene expression changes in white adipose tissue of morbidly obese subjects after surgery-induced weight loss. Diabetes 2005; 54:2277-2286
203. Church TS, Willis MS, Priest EL, LaMonte MJ, Earnest CP, Wilkinson WJ, Wilson DA, Giroir BP. Obesity, macrophage migration inhibitory factor, and weight loss. Int J Obes Relat Metab Disord 2005; 29:675-681
204. Christiansen T, Richelsen B, Bruun JM. Monocyte chemoattractant protein-1 is produced in isolated adipocytes, associated with adiposity and reduced after weight loss in morbid obese subjects. Int J Obes Relat Metab Disord 2004; 29:146-150
205. Glintborg D, Andersen M, Richelsen B, Bruun JM. Plasma monocyte chemoattractant protein-1 and macrophage inflammatory protein-1αare increased in patients with polycystic ovary syndrome and associated with adiposity, but unaffected by pioglitazone treatment. Clin Endocrinol (Oxf) 2009; 71:652-658
206. Permana PA, Menge C, Reaven PD. Macrophage-secreted factors induce adipocyte inflammation and insulin resistance. Biochem Biophys Res Commun 2006; 341:507-514
207. Curat C, Wegner V, Sengenès C, Miranville A, Tonus C, Busse R, Bouloumié A. Macrophages in human visceral adipose tissue: increased accumulation in obesity and a source of resistin and visfatin. Diabetologia 2006; 49:744-747
208. Di Gregorio GB, Yao-Borengasser A, Rasouli N, Varma V, Lu T, Miles LM, Ranganathan G, Peterson CA, McGehee RE, Kern PA. Expression of CD68 and macrophage chemoattractant protein-1 genes in human adipose and muscle tissues: association with cytokine expression, insulin resistance, and reduction by pioglitazone. Diabetes 2005; 54:2305-2313
209. Zeyda M, Stulnig TM. Adipose tissue macrophages. Immunol Lett 2007; 112:61-67
210. Ruan X, Dai Y. Study on chronic low-grade inflammation and influential factors of polycystic ovary syndrome. Med Princ Pract 2009; 18:118-122
211. Diamanti-Kandarakis E, Alexandraki K, Piperi C, Protogerou A, Katsikis I, Paterakis T, Lekakis J, Panidis D. Inflammatory and endothelial markers in women with polycystic ovary syndrome. Eur J Clin Invest 2006; 36:691-697
212. Orio F, Jr., Palomba S, Cascella T, Di Biase S, Manguso F, Tauchmanova L, Nardo LG, Labella D, Savastano S, Russo T, Zullo F, Colao A, Lombardi G. The Increase of Leukocytes as a New Putative Marker of Low-Grade Chronic Inflammation and Early Cardiovascular Risk in Polycystic Ovary Syndrome. J Clin Endocrinol Metab 2005; 90:2-5
213. Tarkun I, Arslan BC, Canturk Z, Turemen E, Sahin T, Duman C. Endothelial Dysfunction in Young Women with Polycystic Ovary Syndrome: Relationship with Insulin Resistance and Low-Grade Chronic Inflammation. J Clin Endocrinol Metab 2004; 89:5592-5596
214. Sayin NC, Gucer F, Balkanli-Kaplan P, Yuce MA, Ciftci S, Kucuk M, Yardim T. Elevated serum TNF-α levels in normal-weight women with polycystic ovaries or the polycystic ovary syndrome. J Reprod Med 2003; 48:165-170
215. Amato G, Conte M, Mazziotti G, Lalli E, Vitolo G, Tucker AT, Bellastella A, Carella C, Izzo A. Serum and Follicular Fluid Cytokines in Polycystic Ovary Syndrome During Stimulated Cycles. Obstet Gynecol 2003; 101:1177-1182

216. Gonzalez F, Thusu K, Abdel-Rahman E, Prabhala A, Tomani M, Dandona P. Elevated serum levels of tumor necrosis factor alpha in normal-weight women with polycystic ovary syndrome. Metabolism 1999; 48:437-441
217. Tosi F, Dorizzi R, Castello R, Maffeis C, Spiazzi G, Zoppini G, Muggeo M, Moghetti P. Body fat and insulin resistance independently predict increased serum C-reactive protein in hyperandrogenic women with polycystic ovary syndrome. Eur J Endocrinol 2009; 161:737-745
218. Kelly CC, Lyall H, Petrie JR, Gould GW, Connell JM, Sattar N. Low Grade Chronic Inflammation in Women with Polycystic Ovarian Syndrome. J Clin Endocrinol Metab 2001; 86:2453-2455
219. Vgontzas AN, Trakada G, Bixler EO, Lin H-M, Pejovic S, Zoumakis E, Chrousos GP, Legro RS. Plasma interleukin 6 levels are elevated in polycystic ovary syndrome independently of obesity or sleep apnea. Metabolism 2006; 55:1076-1082
220. Möhlig M, Spranger J, Osterhoff M, Ristow M, Pfeiffer AF, Schill T, Schlosser HW, Brabant G, Schofl C. The polycystic ovary syndrome per se is not associated with increased chronic inflammation. Eur J Endocrinol 2004; 150:525-532
221. Escobar-Morreale HF, Villuendas G, Botella-Carretero JI, Sancho J, San Millán JL. Obesity, and not insulin resistance, is the major determinant of serum inflammatory cardiovascular risk markers in pre-menopausal women. Diabetologia 2003; 46:625-633
222. Hu WH, Qiao J, Zhao SY, Zhang XW, Li MZ. [Monocyte chemoattractant protein-1 and its correlation with lipoprotein in polycystic ovary syndrome]. Beijing Da Xue Xue Bao 2006; 38:487-491
223. González F, Rote NS, Minium J, Kirwan JP. Evidence of proatherogenic inflammation in polycystic ovary syndrome. Metabolism 2009; 58:954-962
224. Large V, Arner P. Regulation of lipolysis in humans. Pathophysiological modulation in obesity, diabetes, and hyperlipidaemia. Diabetes Metab 1998; 24:409-418
225. Cryer A. Tissue lipoprotein lipase activity and its action in lipoprotein metabolism. Int J Biochem 1981; 13:525-541
226. Lafontan M, Langin D. Lipolysis and lipid mobilization in human adipose tissue. Prog Lipid Res 2009; 48:275-297
227. Moro C, Pasarica M, Elkind-Hirsch K, Redman LM. Aerobic exercise training improves atrial natriuretic peptide and catecholamine-mediated lipolysis in obese women with polycystic ovary syndrome. J Clin Endocrinol Metab 2009; 94:2579-2586
228. Ek I, Arner P, Bergqvist A, Carlstrom K, Wahrenberg H. Impaired Adipocyte Lipolysis in Nonobese Women with the Polycystic Ovary Syndrome: A Possible Link to Insulin Resistance? J Clin Endocrinol Metab 1997; 82:1147-1153
229. Faulds G, Ryden M, Ek I, Wahrenberg H, Arner P. Mechanisms behind lipolytic catecholamine resistance of subcutaneous fat cells in the polycystic ovarian syndrome. J Clin Endocrinol Metab 2003; 88:2269-2273
230. Rosenbaum D, Haber RS, Dunaif A. Insulin resistance in polycystic ovary syndrome: decreased expression of GLUT-4 glucose transporters in adipocytes. Am J Physiol Endocrinol Metab 1993; 264:E197-202
231. Rebuffe-Scrive M, Cullberg G, Lundberg PA, Lindstedt G, Björntorp P. Anthropometric variables and metabolism in polycystic ovarian disease. Horm Metab Res 1989; 21:391-397

232. Lithell H, Nillius SJ, Bergh T, Selinus I. Metabolic profile in obese women with the polycystic ovary syndrome. Int J Obes 1987; 11:1-8
233. Spalding KL, Arner E, Westermark PO, Bernard S, Buchholz BA, Bergmann O, Blomqvist L, Hoffstedt J, Naslund E, Britton T, Concha H, Hassan M, Ryden M, Frisen J, Arner P. Dynamics of fat cell turnover in humans. Nature 2008; 453:783-787
234. Björntorp P, Gustafson A, Persson B. Adipose tissue fat cell size and number in relation to metabolism in endogenous hypertriglyceridemia. Acta Med Scand 1971; 190:363-367
235. Björntorp P, Jonsson A, Berchtold P. Adipose tissue cellularity in maturity onset diabetes mellitus. Acta Med Scand 1972; 191:129-132
236. Krotkiewski M, Sjöstrom L, Björntorp P, Smith U. Regional adipose tissue cellularity in relation to metabolism in young and middle-aged women. Metabolism 1975; 24:703-710
237. Stern MP, Olefsky J, Farquhar JW, Reaven GM. Relationship between fasting plasma lipid levels and adipose tissue morphology. Metabolism 1973; 22:1311-1317
238. Imbeault P, Lemieux S, Prud'homme D, Tremblay A, Nadeau A, Despres JP, Mauriege P. Relationship of visceral adipose tissue to metabolic risk factors for coronary heart disease: is there a contribution of subcutaneous fat cell hypertrophy? Metabolism 1999; 48:355-362
239. Lundgren M, Svensson M, Lindmark S, Renström F, Ruge T, Eriksson J. Fat cell enlargement is an independent marker of insulin resistance and 'hyperleptinaemia'. Diabetologia 2007; 50:625-633
240. Larson-Meyer DE, Heilbronn LK, Redman LM, Newcomer BR, Frisard MI, Anton S, Smith SR, Alfonso A, Ravussin E. Effect of Calorie Restriction With or Without Exercise on Insulin Sensitivity, β-Cell Function, Fat Cell Size, and Ectopic Lipid in Overweight Subjects. Diabetes Care 2006; 29:1337-1344
241. Lönn M, Mehlig K, Bengtsson C, Lissner L. Adipocyte size predicts incidence of type 2 diabetes in women. FASEB J 2010; 24:326-331
242. Weyer C, Foley JE, Bogardus C, Tataranni PA, Pratley RE. Enlarged subcutaneous abdominal adipocyte size, but not obesity itself, predicts type II diabetes independent of insulin resistance. Diabetologia 2000; 43:1498-1506
243. Danforth E, Jr. Failure of adipocyte differentiation causes type II diabetes mellitus? Nat Genet 2000; 26:13
244. Yang X, Jansson P-A, Nagaev I, Jack MM, Carvalho E, Sunnerhagen KS, Cam MC, Cushman SW, Smith U. Evidence of impaired adipogenesis in insulin resistance. Biochem Biophys Res Commun 2004; 317:1045-1051
245. Arner E, Westermark PlO, Spalding KL, Britton T, Rydén M, Frisén J, Bernard S, Arner P. Adipocyte turnover: Relevance to human adipose tissue morphology. Diabetes 2010; 59:105-9
246. Hammarstedt A, Rotter Sopasakis V, Gogg S, Jansson PA, Smith U. Improved insulin sensitivity and adipose tissue dysregulation after short-term treatment with pioglitazone in non-diabetic, insulin-resistant subjects. Diabetologia 2005; 48:96-104
247. Okuno A, Tamemoto H, Tobe K, Ueki K, Mori Y, Iwamoto K, Umesono K, Akanuma Y, Fujiwara T, Horikoshi H, Yazaki Y, Kadowaki T. Troglitazone increases the number of small adipocytes without the change of white adipose tissue mass in obese Zucker rats. J Clin Invest 1998; 101:1354-1361

248. Jacobsson B, Smith U. Effect of cell size on lipolysis and antilipolytic action of insulin in human fat cells. J Lipid Res 1972; 13:651-656
249. Salans LB, Knittle JL, Hirsch J. The role of adipose cell size and adipose tissue insulin sensitivity in the carbohydrate intolerance of human obesity. J Clin Invest 1968; 47:153-165
250. Skurk T, Alberti-Huber C, Herder C, Hauner H. Relationship between Adipocyte Size and Adipokine Expression and Secretion. J Clin Endocrinol Metab 2007; 92:1023-1033
251. Bahceci M, Gokalp D, Bahceci S, Tuzcu A, Atmaca S, Arikan S. The correlation between adiposity and adiponectin, tumor necrosis factor α, interleukin-6 and high sensitivity C-reactive protein levels. Is adipocyte size associated with inflammation in adults? J Endocrinol Invest 2007; 30:210-214
252. Jernås M, Palming J, Sjöholm K, Jennische E, Svensson P-A, Gabrielsson BG, Levin M, Sjögren A, Rudemo M, Lystig TC, Carlsson B, Carlsson LMS, Lönn M. Separation of human adipocytes by size: hypertrophic fat cells display distinct gene expression. FASEB J 2006; 20:1540-1542
253. Sjöholm K, Lundgren M, Olsson M, Eriksson JW. Association of serum amyloid A levels with adipocyte size and serum levels of adipokines: Differences between men and women. Cytokine 2009; 48:260-266
254. Koska J, Stefan N, Dubois S, Trinidad C, Considine RV, Funahashi T, Bunt JC, Ravussin E, Permana PA. mRNA concentrations of MIF in subcutaneous abdominal adipose cells are associated with adipocyte size and insulin action. Int J Obes 2009; 33:842-850
255. Varady KA, Tussing L, Bhutani S, Braunschweig CL. Degree of weight loss required to improve adipokine concentrations and decrease fat cell size in severely obese women. Metabolism 2009; 58:1096-1101
256. Pasarica M, Tchoukalova YD, Heilbronn LK, Fang X, Albu JB, Kelley DE, Smith SR, Ravussin E. Differential Effect of Weight Loss on Adipocyte Size Subfractions in Patients With Type 2 Diabetes. Obesity 2009; 17:1976-1978
257. Löfgren P, Andersson I, Adolfsson B, Leijonhufvud B-M, Hertel K, Hoffstedt J, Arner P. Long-Term Prospective and Controlled Studies Demonstrate Adipose Tissue Hypercellularity and Relative Leptin Deficiency in the Postobese State. J Clin Endocrinol Metab 2005; 90:6207-6213
258. Diamanti-Kandarakis E, Paterakis T, Kandarakis HA. Indices of Low-Grade Inflammation in Polycystic Ovary Syndrome. Ann N Y Acad Sci 2006; 1092:175-186
259. Dunaif A, Segal KR, Shelley DR, Green G, Dobrjansky A, Licholai T. Evidence for distinctive and intrinsic defects in insulin action in polycystic ovary syndrome. Diabetes 1992; 41:1257-1266
260. Eagleson CA, Gingrich MB, Pastor CL, Arora TK, Burt CM, Evans WS, Marshall JC. Polycystic ovarian syndrome: evidence that flutamide restores sensitivity of the gonadotropin-releasing hormone pulse generator to inhibition by estradiol and progesterone. J Clin Endocrinol Metab 2000; 85:4047-4052
261. Elbers JM, Asscheman H, Seidell JC, Megens JA, Gooren LJ. Long-Term Testosterone Administration Increases Visceral Fat in Female to Male Transsexuals. J Clin Endocrinol Metab 1997; 82:2044-2047

262. Moran L, Teede H. Metabolic features of the reproductive phenotypes of polycystic ovary syndrome. Hum Reprod Update 2009; 15:477-488
263. Escobar-Morreale HF, Millan JLS. Abdominal adiposity and the polycystic ovary syndrome. Trends Endocrinol Metab 2007; 18:266-272
264. Joyner J, Hutley L, Cameron D. Intrinsic Regional Differences in Androgen Receptors and Dihydrotestosterone Metabolism in Human Preadipocytes. Horm Metab Res 2002; 34:223-228
265. Arner P. Effects of testosterone on fat cell lipolysis. Species differences and possible role in polycystic ovarian syndrome. Biochimie 2005; 87:39-43
266. Polderman KH, Gooren LJ, Asscheman H, Bakker A, Heine RJ. Induction of insulin resistance by androgens and estrogens. J Clin Endocrinol Metab 1994; 79:265-271
267. Elbers JMH, Giltay EJ, Teerlink T, Scheffer PG, Asscheman H, Seidell JC, Gooren LJ. Effects of sex steroids on components of the insulin resistance syndrome in transsexual subjects. Clin Endocrinol 2003; 58:562-571
268. Diamond MP, Grainger D, Diamond MC, Sherwin RS, DeFronzo RA. Effects of Methyltestosterone on Insulin Secretion and Sensitivity In Women. J Clin Endocrinol Metab 1998; 83:4420-4425
269. Zang H, Carlström K, Arner P, Hirschberg AL. Effects of treatment with testosterone alone or in combination with estrogen on insulin sensitivity in postmenopausal women. Fertil Steril 2006; 86:136-144
270. Moghetti P, Tosi F, Castello R, Magnani CM, Negri C, Brun E, Furlani L, Caputo M, Muggeo M. The insulin resistance in women with hyperandrogenism is partially reversed by antiandrogen treatment: evidence that androgens impair insulin action in women. J Clin Endocrinol Metab 1996; 81:952-960
271. Gambineri A, Patton L, Vaccina A, Cacciari M, Morselli-Labate AM, Cavazza C, Pagotto U, Pasquali R. Treatment with Flutamide, Metformin, and Their Combination Added to a Hypocaloric Diet in Overweight-Obese Women with Polycystic Ovary Syndrome: A Randomized, 12-Month, Placebo-Controlled Study. J Clin Endocrinol Metab 2006; 91:3970-3980
272. Dahlgren E, Landin K, Krotkiewski M, Holm G, Janson PO. Effects of two antiandrogen treatments on hirsutism and insulin sensitivity in women with polycystic ovary syndrome. Hum Reprod 1998; 13:2706-2711
273. Mannerås L, Cajander S, Holmäng A, Seleskovic Z, Lystig T, Lönn M, Stener-Victorin E. A new rat model exhibiting both ovarian and metabolic characteristics of polycystic ovary syndrome. Endocrinology 2007; 148:3781-3791
274. Nilsson C, Niklasson M, Eriksson E, Björntorp P, Holmäng A. Imprinting of Female Offspring with Testosterone Results in Insulin Resistance and Changes in Body Fat Distribution at Adult Age in Rats. J Clin Invest 1998; 101:74-78
275. Alexanderson C, Eriksson E, Stener-Victorin E, Lystig T, Gabrielsson B, Lönn M, Holmäng A. Postnatal testosterone exposure results in insulin resistance, enlarged mesenteric adipocytes, and an atherogenic lipid profile in adult female rats: comparisons with estradiol and dihydrotestosterone. Endocrinology 2007; 148:5369-5376
276. Demissie M, Lazic M, Foecking EM, Aird F, Dunaif A, Levine JE. Transient prenatal androgen exposure produces metabolic syndrome in adult female rats. Am J Physiol Endocrinol Metab 2008; 295:E262-268

277. Dumesic D, Abbott D, Padmanabhan V. Polycystic ovary syndrome and its developmental origins. Rev Endocr Metab Disord 2007; 8:127-141
278. Kamat A, Hinshelwood MM, Murry BA, Mendelson CR. Mechanisms in tissue-specific regulation of estrogen biosynthesis in humans. Trends Endocrinol Metab 2002; 13:122-128
279. Sir-Petermann T, Maliqueo M, Angel B, Lara HE, Perez-Bravo F, Recabarren SE. Maternal serum androgens in pregnant women with polycystic ovarian syndrome: possible implications in prenatal androgenization. Hum Reprod 2002; 17:2573-2579
280. Foecking EM, Szabo M, Schwartz NB, Levine JE. Neuroendocrine consequences of prenatal androgen exposure in the female rat: absence of luteinizing hormone surges, suppression of progesterone receptor gene expression, and acceleration of the gonadotropin-releasing hormone pulse generator. Biol Reprod 2005; 72:1475-1483
281. Ruiz A, Aguilar R, Tebar AM, Gaytan F, Sanchez-Criado JE. RU486-treated rats show endocrine and morphological responses to therapies analogous to responses of women with polycystic ovary syndrome treated with similar therapies. Biol Reprod 1996; 55:1284-1291
282. Kafali H, Iriadam M, Ozardali I, Demir N. Letrozole-induced polycystic ovaries in the rat: A new model for cystic ovarian disease. Arch Med Res 2004; 35:103-108
283. Brawer J, Munoz M, Farookhi R. Development of the polycystic ovarian condition (PCO) in the estradiol valerate-treated rat. Biol Reprod 1986; 35:647-655
284. Baldissera SF, Motta LD, Almeida MC, Antunes-Rodrigues J. Proposal of an experimental model for the study of polycystic ovaries. Braz J Med Biol Res 1991; 24:747-751
285. Beloosesky R, Gold R, Almog B, Sasson R, Dantes A, Land-Bracha A, Hirsh L, Itskovitz-Eldor J, Lessing JB, Homburg R, Amsterdam A. Induction of polycystic ovary by testosterone in immature female rats: Modulation of apoptosis and attenuation of glucose/insulin ratio. Int J Mol Med 2004; 14:207-215
286. Abbott DH, Dumesic DA, Levine JE, Dunaif A, Padmanabhan V. Animal models and fetal programming of the polycystic ovary syndrome. In: Azziz R, Nestler JE, Dewailly D eds, Androgen excess disorders in women: polycystic ovary syndrome and other disorders. Totowa: Human Press Inc; 2006:259-272
287. Hoeger KM. Role of lifestyle modification in the management of polycystic ovary syndrome. Best Pract Res Clin Endocrinol Metab 2006; 20:293-310
288. Hoeger KM, Kochman L, Wixom N, Craig K, Miller RK, Guzick DS. A randomized, 48-week, placebo-controlled trial of intensive lifestyle modification and/or metformin therapy in overweight women with polycystic ovary syndrome: A pilot study. Fertil Steril 2004; 82:421-429
289. Vrbikova J, Cibula D. Combined oral contraceptives in the treatment of polycystic ovary syndrome. Hum Reprod Update 2005; 11:277-291
290. Chen BY, Yu J. Relationship between blood radioimmunoreactive beta-endorphin and hand skin temperature during the electro-acupuncture induction of ovulation. Acupunct Electrother Res 1991; 16:1-5
291. Mo X, Li D, Pu Y, Xi G, Le X, Fu Z. Clinical studies on the mechanism for acupuncture stimulation of ovulation. J Tradit Chin Med 1993; 13:115-119

292. Stener-Victorin E, Waldenström U, Tägnfors U, Lundeberg T, Lindstedt G, Janson PO. Effects of electro-acupuncture on anovulation in women with polycystic ovary syndrome. Acta Obstet Gynecol Scand 2000; 79:180-188
293. Giallauria F, Palomba S, Maresca L, Vuolo L, Tafuri D, Lombardi G, Colao AM, Vigorito C. Exercise training improves autonomic function and inflammatory pattern in women with polycystic ovary syndrome (PCOS). Clin Endocrinol 2008; 69:792-798
294. Randeva HS, Lewandowski KC, Drzewoski J, Brooke-Wavell K, O'Callaghan C, Czupryniak L, Hillhouse EW, Prelevic GM. Exercise decreases plasma total homocysteine in overweight young women with polycystic ovary syndrome. J Clin Endocrinol Metab 2002; 87:4496-4501
295. Vigorito C, Giallauria F, Palomba S, Cascella T, Manguso F, Lucci R, De Lorenzo A, Tafuri D, Lombardi G, Colao A, Orio F. Beneficial Effects of a Three-Month Structured Exercise Training Program on Cardiopulmonary Functional Capacity in Young Women with Polycystic Ovary Syndrome. J Clin Endocrinol Metab 2007; 92:1379-1384
296. Hoeger KM. Exercise therapy in polycystic ovary syndrome. Semin Reprod Med 2008; 26:93-100
297. Clark AM, Ledger W, Galletly C, Tomlinson L, Blaney F, Wang X, Norman RJ. Weight loss results in significant improvement in pregnancy and ovulation rates in anovulatory obese women. Hum Reprod 1995; 10:2705-2712
298. Palomba S, Giallauria F, Falbo A, Russo T, Oppedisano R, Tolino A, Colao A, Vigorito C, Zullo F, Orio F. Structured exercise training programme versus hypocaloric hyperproteic diet in obese polycystic ovary syndrome patients with anovulatory infertility: a 24-week pilot study. Hum Reprod 2008; 23:642-650
299. Ross R, Freeman JA, Janssen I. Exercise alone is an effective strategy for reducing obesity and related comorbidities. Exerc Sport Sci Rev 2000; 28:165-170
300. Kay SJ, Singh MAF. The influence of physical activity on abdominal fat: a systematic review of the literature. Obes Rev 2006; 7:183-200
301. Stener-Victorin E, Jedel E, Janson PO, Sverrisdottir YB. Low-frequency electroacupuncture and physical exercise decrease high muscle sympathetic nerve activity in polycystic ovary syndrome. Am J Physiol Regul Integr Comp Physiol 2009; 297:R387-395
302. Lindmark S, Wiklund U, Bjerle P, Eriksson JW. Does the autonomic nervous system play a role in the development of insulin resistance? A study on heart rate variability in first-degree relatives of Type 2 diabetes patients and control subjects. Diabet Med 2003; 20:399-405
303. Tentolouris N, Argyrakopoulou G, Katsilambros N. Perturbed autonomic nervous system function in metabolic syndrome. Neuromolecular Med 2008; 10:169-178
304. Lund S, Holman GD, Schmitz O, Pedersen O. Contraction stimulates translocation of glucose transporter GLUT4 in skeletal muscle through a mechanism distinct from that of insulin. Proc Natl Acad Sci U S A 1995; 92:5817-5821
305. Douen AG, Ramlal T, Rastogi S, Bilan PJ, Cartee GD, Vranic M, Holloszy JO, Klip A. Exercise induces recruitment of the "insulin-responsive glucose transporter". Evidence for distinct intracellular insulin- and exercise-recruitable transporter pools in skeletal muscle. J Biol Chem 1990; 265:13427-13430

306. Wijesekara J, Thong F, Antonescu CN, Klip A. Diverse signals regulate glucose uptake into skeletal muscle. Can J Diabetes 2006; 30:80-88
307. Ryder JW, Chibalin AV, Zierath JR. Intracellular mechanisms underlying increases in glucose uptake in response to insulin or exercise in skeletal muscle. Acta Physiol Scand 2001; 171:249-257
308. Zierath JR. Exercise Effects of Muscle Insulin Signaling and Action: Invited Review: Exercise training-induced changes in insulin signaling in skeletal muscle. J Appl Physiol 2002; 93:773-781
309. Perseghin G, Price TB, Petersen KF, Roden M, Cline GW, Gerow K, Rothman DL, Shulman GI. Increased Glucose Transport-Phosphorylation and Muscle Glycogen Synthesis after Exercise Training in Insulin-Resistant Subjects. N Engl J Med 1996; 335:1357-1362
310. Andersen P, Henriksson J. Capillary supply of the quadriceps femoris muscle of man: adaptive response to exercise. J Physiol 1977; 270:677-690
311. Laughlin MH, Armstrong RB. Muscular blood flow distribution patterns as a function of running speed in rats. Am J Physiol 1982; 243:H296-306
312. Jansen G, Lundeberg T, Kjartansson J, Samuelson UE. Acupuncture and sensory neuropeptides increase cutaneous blood flow in rats. Neurosci Lett 1989; 97:305-309
313. Dawidson I, Angmar-Mansson B, Blom M, Theodorsson E, Lundeberg T. The influence of sensory stimulation (acupuncture) on the release of neuropeptides in the saliva of healthy subjects. Life Sci 1998; 63:659-674
314. Sato A, Sato Y, Shimura M, Uchida S. Calcitonin gene-related peptide produces skeletal muscle vasodilation following antidromic stimulation of unmyelinated afferents in the dorsal root in rats. Neurosci Lett 2000; 283:137-140
315. Sandberg M, Lundeberg T, Lindberg LG, Gerdle B. Effects of acupuncture on skin and muscle blood flow in healthy subjects. Eur J Appl Physiol 2003; 90:114-119
316. Kniffeki KD, Mense S, Schmidt RF. Muscle receptors with fine afferent fibers which may evoke circulatory reflexes. Circ Res 1981; 48:I25-31
317. Kaufman MP, Waldrop TG, Rybycki KJ, Ordway GA, Mitchell JH. Effects of static and rythmic twitch contractions on the discharge of group III and IV muscle afferents. Cardiovasc Rec 1984; 18:663-668
318. Andersson S, Lundeberg T. Acupuncture - from empiricism to science: functional background to acupuncture effects in pain and disease. Med Hypotheses 1995; 45:271-281
319. Han J. Physiology of Acupuncture: Review of Thirty Years of Research. J Altern Complement Med 1997; 3:s101-s108
320. Stener-Victorin E, Kobayashi R, Kurosawa M. Ovarian blood flow responses to electro-acupuncture stimulation at different frequencies and intensities in anaesthetized rats. Auton Neurosci 2003; 108:50-56
321. Stener-Victorin E, Fujisawa S, Kurosawa M. Ovarian blood flow responses to electroacupuncture stimulation depend on estrous cycle and on site and frequency of stimulation in anesthetized rats. J Appl Physiol 2006; 101:84-91
322. Chao DM, Shen LL, Tjen-A-Looi S, Pitsillides KF, Li P, Longhurst JC. Naloxone reverses inhibitory effect of electroacupuncture on sympathetic cardiovascular reflex responses. Am J Physiol Heart Circ Physiol 1999; 276:H2127-2134

323. Han J. Acupuncture: neuropeptide release produced by electrical stimulation of different frequencies. Trends Neurosci 2003; 26:17-22
324. Bender T, Nagy G, Barna I, Tefner I, Kádas É, Géher P. The effect of physical therapy on beta-endorphin levels. Eur J Appl Physiol 2007; 100:371-382
325. Petti F, Bangrazi A, Liguori A, Reale G, Ippoliti F. Effects of acupuncture on immune response related to opioid-like peptides. J Tradit Chin Med 1998; 18:55-63
326. Clement-Jones V, Tomlin S, Rees L, McLoughlin L, Besser GM, Wen HL. Increased beta-endorphin but not met-enkephalin levels in human cerebrospinal fluid after acupuncture for recurrent pain. Lancet 1980; 316:946-949
327. Hoffmann P, Terenius L, Thorén P. Cerebrospinal fluid immunoreactive β-endorphin concentration is increased by voluntary exercise in the spontaneously hypertensive rat. Regulatory Peptides 1990; 28:233-239
328. Radosevich PM, Nash JA, Lacy DB, O'Donovan C, Williams PE, Abumrad NN. Effects of low- and high-intensity exercise on plasma and cerebrospinal fluid levels of ir-beta-endorphin, ACTH, cortisol, norepinephrine and glucose in the conscious dog. Brain Res 1989; 498:89-98
329. Eyvazzadeh AD, Pennington KP, Pop-Busui R, Sowers M, Zubieta JK, Smith YR. The role of the endogenous opioid system in polycystic ovary syndrome. Fertil Steril 2009; 92:1-12
330. Ferin M, Van Vugt D, Wardlaw S. The hypothalamic control of the menstrual cycle and the role of endogenous opioid peptides. Recent Prog Horm Res 1984; 40:441-485
331. Genazzani AR, Genazzani AD, Volpogni C, Pianazzi F, Li GA, Surico N, Petraglia F. Opioid control of gonadotrophin secretion in humans. Hum Reprod 1993; 8 Suppl 2:151-153
332. Jenkins PJ, Grossman A. The control of the gonadotrophin releasing hormone pulse generator in relation to opioid and nutritional cues. Hum Reprod 1993; 8 Suppl 2:154-161
333. Chan JS, Lu CL, Seidah NG, Chretien M. Corticotropin releasing factor (CRF): effects on the release of pro-opiomelanocortin (POMC)-related peptides by human anterior pituitary cells in vitro. Endocrinology 1982; 111:1388-1390
334. Bruni JF, Watkins WB, Yen SSC. Beta-endorphin in the human pancreas. J Clin Endocrinol Metab 1979; 49:649-651
335. Curry DL, Bennett LL, Li CH. Stimulation of insulin secretion by beta-endorphins (1-27 & 1-31). Life Sci 1987; 40:2053-2058
336. Givens JR, Wiedemann E, Andersen RN, Kitabchi AE. beta-Endorphin and beta-lipotropin plasma levels in hirsute women: correlation with body weight. J Clin Endocrinol Metab 1980; 50:975-976
337. Martinez-Guisasola J, Guerrero M, Alonso F, Diaz F, Cordero J, Ferrer J. Plasma beta-endorphin levels in obese and non-obese patients with polycystic ovary disease. Gynecol Endocrinol 2001; 15:14-22
338. Ahmed MI, Duleba AJ, El Shahat O, Ibrahim ME, Salem A. Naltrexone treatment in clomiphene resistant women with polycystic ovary syndrome. Hum Reprod 2008; 23:2564-2569

339. Fruzzetti F, Bersi C, Parrini D, Ricci C, Genazzani AR. Effect of long-term naltrexone treatment on endocrine profile, clinical features, and insulin sensitivity in obese women with polycystic ovary syndrome. Fertil Steril 2002; 77:936-944
340. Hadziomerovic D, Rabenbauer B, Wildt L. Normalization of hyperinsulinemia by chronic opioid receptor blockade in hyperandrogenemic women. Fertil Steril 2006; 86:651-657
341. Fulghesu AM, Ciampelli M, Guido M, Murgia F, Caruso A, Mancuso S, Lanzone A. Role of opioid tone in the pathophysiology of hyperinsulinemia and insulin resistance in polycystic ovarian disease. Metabolism 1998; 47:158-162
342. Genazzani AR, Facchinetti F, Petraglia F, Pintor C, Corda R. Hyperendorphinemia in obese children and adolescents. J Clin Endocrinol Metab 1986; 62:36-40
343. Cabioglu MT, Ergene N. Electroacupuncture therapy for weight loss reduces serum total cholesterol, triglycerides, and LDL cholesterol levels in obese women. Am J Chin Med 2005; 33:525-533
344. Cabioglu MT, Ergene N. Changes in levels of serum insulin, C-Peptide and glucose after electroacupuncture and diet therapy in obese women. Am J Chin Med 2006; 34:367-376
345. Cabioglu MT, Ergene N. Changes in serum leptin and beta endorphin levels with weight loss by electroacupuncture and diet restriction in obesity treatment. Am J Chin Med 2006; 34:1-11
346. Hsu CH, Hwang KC, Chao CL, Lin JG, Kao ST, Chou P. Effects of electroacupuncture in reducing weight and waist circumference in obese women: a randomized crossover trial. Int J Obes Relat Metab Disord 2005; 29:1379-1384
347. Cho SH, Lee JS, Thabane L, Lee J. Acupuncture for obesity: a systematic review and meta-analysis. Int J Obes 2009; 33:183-196
348. Wang SJ, Li Q, She YF, Li AY, Xu HZ, Zhao ZG. [Effect of electroacupuncture on metabolism of lipids in rats of obesity induced by sodium glutamate]. Zhongguo Zhen Jiu 2005; 25:269-271
349. Franks S. Adult polycystic ovary syndrome begins in childhood. Best Pract Res Clin Endocrinol Metab 2002; 16:263-272
350. Apter D, Butzow T, Laughlin GA, Yen SS. Accelerated 24-hour luteinizing hormone pulsatile activity in adolescent girls with ovarian hyperandrogenism: relevance to the developmental phase of polycystic ovarian syndrome. J Clin Endocrinol Metab 1994; 79:119-125
351. Ibanez L, de Zegher F, Potau N. Premature pubarche, ovarian hyperandrogenism, hyperinsulinism and the polycystic ovary syndrome: from a complex constellation to a simple sequence of prenatal onset. J Endocrinol Invest 1998; 21:558-566
352. Chakrabarty S, Miller BT, Collins TJ, Nagamani M. Ovarian Dysfunction in Peripubertal Hyperinsulinemia. J Soc Gynecol Investig 2006; 13:122-129
353. Fassnacht M, Schlenz N, Schneider SB, Wudy SA, Allolio B, Arlt W. Beyond adrenal and ovarian androgen generation: increased peripheral 5α-reductase activity in women with polycystic ovary syndrome. J Clin Endocrinol Metab 2003; 88:2760-2766
354. Silfen ME, Denburg MR, Manibo AM, Lobo RA, Jaffe R, Ferin M, Levine LS, Oberfield SE. Early endocrine, metabolic, and sonographic characteristics of polycystic ovary

syndrome (PCOS): comparison between nonobese and obese adolescents. J Clin Endocrinol Metab 2003; 88:4682-4688
355. Shyu BC, Andersson SA, Thoren P. Spontaneous running in wheels. A microprocessor assisted method for measuring physiological parameters during exercise in rodents. Acta Physiol Scand 1984; 121:103-109
356. Hatch R, Rosenfield RL, Kim MH, Tredway D. Hirsutism: implications, etiology, and management. Am J Obstet Gynecol 1981; 140:815-830
357. Marcondes FK, Bianchi FJ, Tanno AP. Determination of the estrous cycle phases of rats: Some helpful considerations. Braz J Biol 2002; 62:609-614
358. Goodpaster BH. Measuring body fat distribution and content in humans. Curr Opin Clin Nutr Metab Care 2002; 5:481-487
359. Abate N, Burns D, Peshock R, Garg A, Grundy S. Estimation of adipose tissue mass by magnetic resonance imaging: validation against dissection in human cadavers. J Lipid Res 1994; 35:1490-1496
360. Kullberg J, Ahlström H, Johansson L, Frimmel H. Automated and reproducible segmentation of visceral and subcutaneous adipose tissue from abdominal MRI. Int J Obes 2007; 31:1806-1817
361. DeFronzo RA, Tobin JD, Andres R. Glucose clamp technique: a method for quantifying insulin secretion and resistance. Am J Physiol Endocrinol Metab 1979; 237:E214-223
362. Matthews DR, Hosker JP, Rudenski AS, Naylor BA, Treacher DF, Turner RC. Homeostasis model assessment: insulin resistance and β-cell function from fasting plasma glucose and insulin concentrations in man. Diabetologia 1985; 28:412-419
363. Katz A, Nambi SS, Mather K, Baron AD, Follmann DA, Sullivan G, Quon MJ. Quantitative Insulin Sensitivity Check Index: A Simple, Accurate Method for Assessing Insulin Sensitivity In Humans. J Clin Endocrinol Metab 2000; 85:2402-2410
364. Skrha J, Haas T, Sindelka G, Prazny M, Widimsky J, Cibula D, Svacina S. Comparison of the Insulin Action Parameters from Hyperinsulinemic Clamps with Homeostasis Model Assessment and QUICKI Indexes in Subjects with Different Endocrine Disorders. J Clin Endocrinol Metab 2004; 89:135-141
365. Sjöstrom L, Björntorp P, Vrana J. Microscopic fat cell size measurements on frozen-cut adipose tissue in comparison with automatic determinations of osmium-fixed fat cells. J Lipid Res 1971; 12:521-530
366. Björnheden T, Jakubowicz B, Levin M, Oden B, Eden S, Sjöström L, Lönn M. Computerized determination of adipocyte size. Obes Res 2004; 12:95-105
367. Goldrick RB. Morphological changes in the adipocyte during fat deposition and mobilization. Am J Physiol 1967; 212:777-782
368. Applied-Biosystems. Application note: amplification efficiency of TaqMan® gene expression assays. Applied Biosystems, Foster City CA, USA 2006
369. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2-ΔΔCT method. Methods 2001; 25:402-408
370. Huggett J, Dheda K, Bustin S, Zumla A. Real-time RT-PCR normalisation; strategies and considerations. Genes Immun 2005; 6:279-284
371. Andersen CL, Jensen JL, Ørntoft TF. Normalization of real-time quantitative reverse transcription-PCR data: a model-based variance estimation approach to identify

genes suited for normalization, applied to bladder and colon cancer data sets. Cancer Res 2004; 64:5245-5250
372. Bustin SA, Nolan T. Pitfalls of quantitative real-time reverse-transcription polymerase chain reaction. J Biomol Tech 2004; 15:155-166
373. Ruge T, Svensson M, Eriksson JW, Olivecrona G. Tissue-specific regulation of lipoprotein lipase in humans: effects of fasting. Eur J Clin Invest 2005; 35:194-200
374. Vermeulen A, Verdonck L, Kaufman JM. A Critical Evaluation of Simple Methods for the Estimation of Free Testosterone in Serum. J Clin Endocrinol Metab 1999; 84:3666-3672
375. van den Beld AW, de Jong FH, Grobbee DE, Pols HA, Lamberts SW. Measures of Bioavailable Serum Testosterone and Estradiol and Their Relationships with Muscle Strength, Bone Density, and Body Composition in Elderly Men. J Clin Endocrinol Metab 2000; 85:3276-3282
376. Stener-Victorin E, Holm G, Labrie F, Nilsson L, Janson PO, Ohlsson C. Are There Any Sensitive and Specific Sex Steroid Markers for Polycystic Ovary Syndrome? J Clin Endocrinol Metab 2009 Dec 16 [Epub ahead of print]
377. Conovor WJ. Practical Nonparametric Statistics. 1st ed. New York: John Wiley & Sons Inc; 1971:309-314
378. Perello M, Castrogiovanni D, Giovambattista A, Gaillard RC, Spinedi E. Impairment in insulin sensitivity after early androgenization in the post-pubertal female rat. Life Sci 2007; 80:1792-1798
379. Pasquali R. Obesity and androgens: facts and perspectives. Fertil Steril 2006; 85:1319-1340
380. Blouin K, Boivin A, Tchernof A. Androgens and body fat distribution. J Steroid Biochem Mol Biol 2008; 108:272-280
381. Webber LJ, Stubbs SA, Stark J, Margara RA, Trew GH, Lavery SA, Hardy K, Franks S. Prolonged Survival in Culture of Preantral Follicles from Polycystic Ovaries. J Clin Endocrinol Metab 2007; 92:1975-1978
382. Mayes JS, Watson GH. Direct effects of sex steroid hormones on adipose tissues and obesity. Obes Rev 2004; 5:197-216
383. Brzechffa PR, Jakimiuk AJ, Agarwal SK, Weitsman SR, Buyalos RP, Magoffin DA. Serum immunoreactive leptin concentrations in women with polycystic ovary syndrome. J Clin Endocrinol Metab 1996; 81:4166-4169
384. Blouin K, Nadeau M, Perreault M, Veilleux A, Drolet R, Marceau P, Mailloux J, Luu-The V, Tchernof A. Effects of androgens on adipocyte differentiation and adipose tissue explant metabolism in men and women. Clin Endocrinol (Oxf) 200 Jun 2 [Epub ahead of print]
385. Borggreve SE, Vries Rd, Dullaart RPF. Alterations in high-density lipoprotein metabolism and reverse cholesterol transport in insulin resistance and type 2 diabetes mellitus: role of lipolytic enzymes, lecithin:cholesterol acyltransferase and lipid transfer proteins. Eur J Clin Invest 2003; 33:1051-1069
386. Ricquier D, Bouillaud F. Mitochondrial uncoupling proteins: from mitochondria to the regulation of energy balance. J Physiol 2000; 529:3-10
387. Dietze-Schroeder D, Sell H, Uhlig M, Koenen M, Eckel J. Autocrine action of adiponectin on human fat cells prevents the release of insulin resistance-inducing factors. Diabetes 2005; 54:2003-2011

388. Dietze D, Koenen M, Röhrig K, Horikoshi H, Hauner H, Eckel J. Impairment of insulin signaling in human skeletal muscle cells by co-culture with human adipocytes. Diabetes 2002; 51:2369-2376
389. Manni L, Cajander S, Lundeberg T, Naylor AS, Aloe L, Holmäng A, Jonsdottir IH, Stener-Victorin E. Effect of exercise on ovarian morphology and expression of nerve growth factor and α_1- and β_2-adrenergic receptors in rats with steroid-Induced polycystic ovaries. J Neuroendocrinol 2005; 17:846-858
390. Feng Y, Johansson J, Shao R, Mannerås L, Fernandez-Rodriguez J, Billig H, Stener-Victorin E. Hypothalamic Neuroendocrine Functions in Rats with Dihydrotestosterone-Induced Polycystic Ovary Syndrome: Effects of Low-Frequency Electro-Acupuncture. PLoS ONE 2009; 4:e6638
391. Mannerås L, Cajander S, Lönn M, Stener-Victorin E. Acupuncture and exercise restore adipose tissue expression of sympathetic markers and improve ovarian morphology in rats with dihydrotestosterone-induced PCOS. Am J Physiol Regul Integr Comp Physiol 2009; 296:R1124-1131
392. Barria A, Leyton V, Ojeda SR, Lara HE. Ovarian steroidal response to gonadotropins and beta-adrenergic stimulation is enhanced in polycystic ovary syndrome: role of sympathetic innervation. Endocrinology 1993; 133:2696-2703
393. Stener-Victorin E, Lundeberg T, Cajander S, Aloe L, Manni L, Waldenström U, Janson P. Steroid-induced polycystic ovaries in rats: effect of electro-acupuncture on concentrations of endothelin-1 and nerve growth factor (NGF), and expression of NGF mRNA in the ovaries, the adrenal glands, and the central nervous system. Reprod Biol Endocrinol 2003; 1:33
394. Stener-Victorin E, Lundeberg T, Waldenström U, Manni L, Aloe L, Gunnarsson S, Janson PO. Effects of electro-acupuncture on nerve growth factor and ovarian morphology in rats with experimentally induced polycystic ovaries. Biol Reprod 2000; 63:1497-1503