Polycystic ovary syndrome

Effect of acupuncture on insulin resistance and neuroendocrine function

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"*Jag vet inte vart jag ska, men jag är på väg*"

Carl Sagan

ABSTRACT

Although polycystic ovary syndrome (PCOS) is the most common endocrine disorder amongst women in reproductive age the etiology and pathophysiology are poorly understood. PCOS is characterized by hyperandrogenism, polycystic ovaries and ovulatory dysfunction. It is also associated with metabolic disturbances, increased luteinizing hormone (LH) secretion and increased muscle nerve sympathetic activity. Acupuncture with combined electrical (EA) and manual needle stimulation has been demonstrated to improve menstrual frequencies and to reduce androgen and glucuronidated androgen metabolite levels in women with PCOS. In a dihydrotestosterone (DHT) induced rat PCOS model EA has been shown to improve insulin sensitivity, decrease markers of sympathetic activity in adipose tissue and to improve ovarian morphology.

The overall aims of this thesis were to evaluate the effect of acupuncture on ovulatory and neuroendocrine as well as metabolic dysfunction in women with PCOS and in rats with DHT-induced PCOS, and to search for potential molecular mechanisms mediating the effects. In the rat model we also sought to compare acupuncture with manual and electrical needle stimulation with regards to their efficacy and signaling mechanisms on glucose regulation.

EA 5 days per week during 4-5 weeks in DHT-induced PCOS rats restored estrous cyclicity and reduced elevated protein expression of hypothalamic gonadotropin releasing hormone (GnRH) and androgen receptor (AR). Immunohistochemistry also revealed a co-localization between the two, indicative of AR activation as a mediator of the effects. PCOS women were randomly allocated to either acupuncture with combined electrical and manual stimulation or attention control twice weekly for 10-13 wks. Ovulation frequency was higher in the acupuncture than in the control group, but was not accompanied by changes in LH or cortisol secretion patterns. Furthermore, most sex steroids; estrogens, androgens and androgen precursors and glucuronidated androgen metabolites decreased in the acupuncture group and differed from the control group. The effect on ovulatory function has now repeatedly been shown in both clinical and experimental studies. Here it appears to be related to regulation of sex steroids rather than gonadotropin secretion in women with PCOS although the rat data indicates a relation to normalization of hypothalamic aberrations after EA treatment. EA 5 days per week during 4-5 weeks normalized insulin sensitivity and increased low plasma membrane glucose transporter 4 content in

skeletal muscle of DHT-induced PCOS rats while glucose tolerance was partly improved after manual stimulation. Manual stimulation primarily affected gene expression while electrical stimulation primarily affected protein expression, indicating different mechanisms of action. This suggests that treatment frequency and stimulation modality is of importance and that electrical stimulation of the needles is superior to manual stimulation although this needs to be investigated in clinic.

As shown in this thesis, acupuncture treatment elicits local and systemic effects which have the capacity to break the vicious circle of androgen excess, ovarian dysfunction and possibly reduced insulin sensitivity in PCOS. It may therefore represent an alternative or compliment to standard pharmacological or surgical treatment.

SAMMANFATTNING PÅ SVENSKA

Polycystiskt ovariesyndrom (PCOS) är den vanligaste hormonella störningen hos kvinnor i fertil ålder. Det drabbar ungefär var tionde kvinna och påverkar både reproduktiv och metabol funktion. Dessa kvinnor har ökade nivåer av det manliga könshormonet testosteron vilket kan ge symptom som ökad kroppsbehåring och acne. De har även oregelbunden eller helt utebliven ägglossning vilket kan medföra fertilitetsproblem. De metabola störningarna innefattar okänslighet för insulin samt en högre benägenhet för övervikt/fetma. Tillsammans medför detta en högre risk för typ 2-diabetes och hjärt-kärlsjukdomar.

Då det i nuläget inte finns någon bot omfattar behandlingen istället medicinsk eller kirurgisk lindring av de olika symptomen, vilket ofta involverar biverkningar. Tidigare studier har visat att akupunktur har positiva effekter på både reproduktiv och metabol funktion med få biverkningar.

Syftet med denna avhandling var att utvärdera effekten av akupunktur på ägglossning och reglering av hormoner från hjärnan (hypothalamus/hypofys), äggstockar och binjurar, samt på metabol funktion hos kvinnor med PCOS och hos råttor med PCOS som utvecklats genom kontinuerlig tillförsel av manligt könshormon. Ytterligare ett syfte var att studera möjliga bakomliggande mekanismer i hypothalamus/hypofys avseende reglering av ägglossning samt i muskulatur och fett för reglering av metabol funktion som kan förklara effekten av behandling med akupunktur vid PCOS.

I en randomiserad klinisk studie studerades effekten av akupunktur på reglering av ägglossning samt utsöndring av hormoner från hjärna, äggstockar och binjurar. Kontrollgruppen träffade en terapeut samma antal gånger men utan akupunkturbehandling för att kontrollera för det terapeutiska mötet och tiden det innefattar men utan nålinsättning. Akupunkturgruppen hade 28% högre ägglossningsfrekvens jämfört med kontrollgruppen. Akupunktur sänkte höga nivåer hormoner frisatta från äggstockar och binjurar, samt inhibin B, ett hormon som produceras av de växande äggfolliklarna och kan påverka deras hormonproduktion, utan någon effekt på hormon som frisätts från hjärnan. Råttor med PCOS fick också mer regelbunden ägglossning. Denna effekt kan tänka förklaras av ett samtidigt förändrat uttryck av vissa proteiner i hjärnan som reglerar ägglossning.

Effekten av akupunktur på metabol funktion hos råttor med PCOS visade på en normalisering av insulinkänslighet efter elektrisk stimulering av nålarna, vilken ger

muskelsammandragningar, samt en delvis förbättrad glukostolerans efter akupunktur med manuell stimulering av nålarna. Dessa effekter verkar styras av olika mekanismer, med förändrat proteinutryck främst i muskulatur efter akupunktur med elektrisk stimulering. Våra resultat indikerar även att elektrisk stimulering är mer effektiv än manuell, även om vi inte kan utesluta att olikheterna beror på skillnader i behandlingstid då manuell stimulering utfördes var femte minut och den elektriska är kontinuerlig.

Sammantaget visar fynden i denna avhandling att akupunktur är fördelaktigt för de reproduktiva problemen i PCOS, något som vi demonstrerat i både kliniska och djurexperimentella studier. Betydelsen bör undersökas i jämförande studier mot nuvarande behandling innan akupunktur kan betraktas som ett eventuellt behandlingsalternativ. I de djurexperimentella studierna har vi även sett en upprepad positiv effekt på metabol funktion, något som bör överföras och undersökas vidare i klinik.

LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I. **Hypothalamic neuroendocrine functions in rats with dihydrotestosterone-induced polycystic ovary syndrome: Effects of low-frequency electro-acupuncture.** Feng Yi, Julia Johansson, Ruijin Shao, Louise Mannerås, Julia Fernandez-Rodriguez, Håkan Billig, and Elisabet Stener-Victorin. *PloS ONE 2009, 4(8): e6638.*

II. **Acupuncture for ovulation induction in polycystic ovary syndrome: A randomized controlled trial.** Julia Johansson, Leanne Redman, Paula P Veldhuis, Antonina Sazonova, Fernand Labrie, Göran Holm, Gudmundur Johannsson, and Elisabet Stener-Victorin. *Submitted*

III. **Intense electroacupuncture normalizes insulin sensitivity, increases muscle GLUT4 content, and improves lipid profile in a rat model of polycystic ovary syndrome.** Julia Johansson, Feng Yi, Ruijin Shao, Malin Lönn, Håkan Billig, and Elisabet Stener-Victorin. *Am J Physiol Endocrinol Metab 2010, 299:E551–E559.*

IV. **Electrical vs manual acupuncture stimulation in a rat model of polycystic ovary syndrome: Different effects on muscle and fat tissue insulin signaling.** Julia Johansson, Louise Mannerås-Holm, Ruijin Shao, AnneLiese Olsson, Malin Lönn, Håkan Billig, and Elisabet Stener-Victorin, *PLoS ONE 2013, 8(1): e54357.*

Contents

ABBREVIATIONS

ACTH   Adrenocorticotropic hormone

ADTG   Androsterone glucoronide

AD3G   Androstane-3α, 17β-diol-3glucoronide

AD17G   Androstane-3α, 17β-diol-17glucoronide

AMPK   5' adenosine monophosphate-activated protein kinase

ANOVA   Analysis of variance

ASRM   American society for reproductive medicine

BMI   Body mass index

CGRP   Calcitonin gene-related peptide

COC   Combined oral contraceptives

CRH   Corticotropin releasing hormone

C^T Cycle threshold

CVD   Cardiovascular disease

CYP19a1   Cytochrome P450 aromatase

DAB   3,3'-Diaminobenzidine

DHEA   Dehydroepiandrosterone

DHEA-S   Dehydroepiandrosterone sulphate

DHT   5α-dihydrotestosterone

E_1 Estrone

E_2 Estradiol

EA	Electro-acupuncture
ELISA	Enzyme-linked immunosorbent assay
ESHRE	European Society for Human Reproduction and Embryology
FG	Ferriman Gallwey
FSH	Follicle stimulating hormone
GC	Gas chromatography
GnRH	Gonadotropin releasing hormone
GnRH-ir	Gonadotropin releasing hormone immunoreactive
HPO	Hypothalamic-pituitary-ovarian
HOMA	Homeostasis model assessment
HDB	Horizontal limb of the diagonal band
IHC	Immunohistochemistry
IR	Insulin receptor
LDA	Low density array
LH	Luteinizing hormone
MPO	Medial preoptic area
MRI	Magnetic resonance imaging
MS	Mass spectrometry
NIH	National institutes of health
NGF	Nerve growth factor
NPY	Neuropeptide Y
OGTT	Oral glucose tolerance test

PCO	Polycystic ovaries
PCOS	Polycystic ovary syndrome
PCR	Polymerase chain reaction
POMC	Pro-opiomelanocortin
PVDF	Polyvinylidene difluoride
R	Receptor
RCT	Randomized controlled study
RIA	Radioimmunoassay
SHBG	Sex hormone binding globulin
RT-PCR	Real-time polymerase chain reaction
UPLC	Ultra performance liquid chromatography
VIP	Vasoactive intestinal polypeptide
VMH	Ventromedial hypothalamus
WHR	Waist-hip-ratio
WHO	World Health Organization
QUICKI	Quantitative insulin sensitivity check index
4-DIONE	Androstenedione

INTRODUCTION

Polycystic ovary syndrome

Historically, good health has been a pillar stone for survival and reproduction, and womanhood has always been closely related to the unique childbearing capacity. Infertility is defined as the inability to conceive after two years of regular trying without contraception, and according to World Health Organization (WHO) up to 15% of reproductive-aged couples are affected. However, apart from at the clinic, this is often concealed in the private sphere of the home. Ovulatory dysfunction represent a large part of female infertility. Alongside, prevalence of obesity and the associated metabolic syndrome are at constant rise in the modern world. All these factors, together with abnormally high levels of androgens, are prevalent findings in the female polycystic ovary syndrome (PCOS), a syndrome with psychological, social and economic consequences.

Worldwide, PCOS is the most common endocrine disorder among women of reproductive age. It was first described as early as 1935 by Stein and Leventhal and is a multifactorial disorder characterized by the co-existence of hyperandrogenism, ovulatory dysfunction and, which has given it its name, polycystic ovaries (PCO) (1, 2). Since then, large efforts has been placed on finding a cause, but also on agreeing on a definition and treatment alternatives. The more recent knowledge that women with PCOS also are susceptible to the metabolic syndrome assigned it an increased level of attention (3, 4).

Definition and prevalence

PCOS is defined as a syndrome; hence a single diagnostic criterion cannot solely be the subject of diagnosis. It is also a diagnosis of exclusion, meaning that symptoms clearly derived from other etiologies should be excluded. Although it has been recognized for more than 70 years there is no cohesive definition, and the diagnosis is controversial and still causes debate. The most recent and used definitions are the Rotterdam, NIH and AES criteria (Table 1). First out was the NIH criteria, agreed on in 1990 during an expert conference held at the National Institutes of Health (NIH). This definition includes both ovulatory dysfunction (oligo- or anovulation) and hyperandrogenism (biochemical or the clinical signs; acne or hirsutism) (5). In 2003, on the Rotterdam conference sponsored by European Society for Human

Reproduction and Embryology (ESHRE) and American Society for Reproductive Medicine (ASRM), the definition was broadened by the addition of polycystic ovaries. Two out of three criteria should be fulfilled for diagnosing PCOS (6). In 2006 the definition was again confined by the Androgen Excess and PCOS Society (AES) who required androgen excess as a *fundamental* component in the diagnosis, together with the addition of at least one of the other components for diagnosis (7).

Table 1. *Diagnostic criteria of PCOS.*

Diagnostic criteria	NIH 1990	Rotterdam 2003	AES 2006
	Require:	At least two out of:	Require:
Hyperandrogenism (HA)[A]	√	√	√
Ovulatory dysfunction (OD)[B]	√	√	and/or √
PCO morphology (PCO)[C]		√	and/or √
Possible phenotypes:	1. HA+OD	1. HA+OD+PCO 2. HA+OD 3. HA+PCO 4. PCO+OD	1. HA+OD+PCO 2. HA+OD 3. HA+PCO

[A] Clinical or biochemical signs of hyperandrogenism.
[B] Oligomennorhea, amenorrhea, oligoovulation, or anovulation.
[C] ≥12 follicles of 2–9 mm and/or enlarged ovarian volume of ≥10 mL in one or both ovaries.

All three definitions are presently used in clinic, however many cases remain undiagnosed. In consequence prevalence is difficult to conclude and depends on the used definition, as well as the ethnicity of the measured population. This year a prevalence study of 527 females from Ankara concluded that the prevalence using the NIH, Rotterdam and AES criteria were 6.1, 19.9 and 15.3% respectively (8). A twice as large Australian study by March et al in 2010 concluded prevalence up to 8.7, 17.8 and 12.0% respectively, that is, rather similar numbers (9). Other reports claim prevalence of between 6-10% with the NIH and up to 20% with the Rotterdam criteria (10).

The different definitions result in several PCOS phenotypes (Table 1) with a range of severity of the syndrome. In classical PCOS, when all three criteria are met, women have a metabolically more unhealthy profile than ovulatory PCOS women and the mildest form of normo-androgenemic PCOS. The range of this comprises concerns when comparing clinical studies using different definitions. After a recent NIH workshop on PCOS it was recommended to explicitly report the specific phenotypes in all future clinical studies (11, 12).

Clinical presentations

Hyperandrogenism

The most common feature of PCOS, elevated androgen levels, affects around 60-80% of PCOS women and can result in the clinical signs; hirsutism, acne, and to some extent alopecia (male pattern baldness) (7). Hirsutism is the excess of body hair, with a typical male pattern, and can be self-evaluated with a modified Ferriman-Gallwey scoring system where a score of eight or above signify hirsutism (13). The prevalence of hirsutism differs between ethnic populations but is present in 40-92% of PCOS women (14). Although acne and alopecia are recognized as signs of PCOS, they are observed less frequent than hirsutism, and the relations to hyperandrogenism have been more questioned (1, 7, 14). Hyperandrogenism is neither necessarily always the cause of hirsutism and acne, hence other possible reasons must be excluded. Biochemical markers of hyperandrogenism can therefore be useful in the diagnosis of PCOS (7).

Menstrual irregularities

Menstrual irregularities or oligo-/amennorhea are indicators of, but not equal to, oligo-/anovulation. Oligomenorrhea is usually defined as a cycle length of > 35 days whereas in amennorhea the intercycle interval exceeds 90 days. The prevalence of menstrual irregularities in PCOS depends on the used diagnostic criteria but is approximately 75% (7). If using the NIH criteria, of course all patients will experience menstrual irregularities. Ultimately, irregular ovulation can cause infertility due to difficulties conceiving, and should be acknowledged.

Obesity

Although metabolic dysfunction is not a part of the diagnosis the prevalence of women with PCOS being either overweight or obese is high (15). Studies show a great variety between populations, and the highest prevalence's were found in large studies from Australia and United states where up to 85% of women with PCOS were overweight or obese (16, 17). More wide estimates are somewhere between 38% and 88% (18). PCOS is also strongly associated with insulin resistance, hyperinsulinemia, type 2 diabetes and dyslipidemia (1). PCOS is often said to be associated with central obesity and although excess body weight, especially with central or upper body fat distribution, has been shown to increase both the metabolic and reproductive symptoms of PCOS, the literature is somewhat inconsistent (15). The first study of visceral and abdominal fat mass distribution made by magnetic resonance imaging (MRI) could not discriminate PCOS from BMI matched controls although insulin sensitivity was impaired in the PCOS group, indicating functional differences rather than positional in the adipose tissue of PCOS women (19). This study may be criticized due to age differences, but was later supported by our own previous MRI measurements with weight- and age pair-wise matched PCOS and controls (20). The same year, though, yet another study of not more than 10 lean women with PCOS showed decreased visceral fat mass, whilst the most recent study show increased visceral abdominal fat (21, 22). Moreover, there is a need of a standardization of the anatomical positioning of the abdominal images, since there is now a large variation between studies (23). In summary, the MRI results on visceral adiposity in PCOS are inconclusive but this may be due to differences in BMI, PCOS definition or anatomical positioning of the MRI sections and larger standardized studies should be performed.

Pathophysiology and etiology of PCOS

Despite the high incidence, the etiology of PCOS remains unknown. Due to the heterogeneity in the representation of clinical and biochemical features it has been debated whether it actually represents one single disorder or several. Symptoms of PCOS often manifest around puberty, but the origin may be programmed already as early as during fetal development (24-26). Androgens possess a central position in PCOS as is closely related to the ovarian morphology and they are sufficient to cause

PCOS like states in both animal models and female-to-male transsexuals (27, 28). It has therefore been presented as the likely principal cause of PCOS. Since it is not known when or where the pathology actually begins, several different hypotheses are presented. Prenatal androgenization represents an established hypothesis of PCOS etiology and is based on animal models including monkeys, sheep and rodents where prenatal androgenization inflicts several features of PCOS in the offspring (29-32). However, in humans, although increased levels of androgens have been found in pregnant PCOS women (33), only one study has found increased levels of testosterone in umbilical vein blood in infants of PCOS mothers although these levels were measured by immunoassays and not mass spectrometry (34). This study had though a number of limitations such as small sample size and deficient testosterone detection method. On the other hand, a larger prospective study could not demonstrate any association between maternal and umbilical cord blood, nor a relationship to PCOS (35). However, also this study admits limitations. No measurements of placental aromatase activity were made and the late timing of measurements might not reflect earlier sensitive periods for androgen exposure. They also used adolescent females, whose diagnoses are more problematic (36, 37). The latest study where androgens in both maternal and fetal circulation were measured with mass spectrometry a relation between the maternal testosterone levels and cord blood testosterone was found in male offspring, but not in female. However, maternal insulin levels was associated with higher androgen levels in the fetus (38). Prepubertal exposure to androgens is yet another hypothesis that originates in the pubertal start of symptom manifestation (25, 26), from which several animal models of PCOS has been developed (39-41).

Although not a part of the diagnosis, PCOS is also frequently associated with insulin resistance and compensatory hyperinsulinemia (42). Both androgens and insulin, which both increase during puberty, are therefore considered to be two key players in PCOS and long-standing is the feud concerning which one of these that is related to the etiology (25, 26).

Asides from these, PCOS characteristics such as dysfunctional regulation of gonadotropins, intraovarian factors causing altered ovarian steroidogenesis, altered adrenal androgen production, increased sympathetic activity, and genetics are proposed as causative mechanisms. Genetic studies of PCOS are challenging due to the complexity and heterogeneity of the disorder. Studies of heritability show a familial aggregation of cases and confirm a strong genetic component but also unique environmental influences (43, 44). There are also increased prevalence's of

hyperandrogenemia, metabolic dysfunctions and type-2-diabetes, in first-degree relatives of women with PCOS, with PCO morphology being a marker of the endocrine and metabolic characteristics (45-47). The etiology may therefore involve a genetic component, predisposing some women to PCOS. Gene association studies has until now focused on genes involved in TGF-β pathway, insulin signaling or with relation to obesity or type 2 diabetes, but there is now more and more focus on genome-wide association studies although they demand much larger data sets (44).

Androgen excess

Androgens in females are produced both in the ovaries and the adrenals and include dehydroepiandrosterone sulphate (DHEA-S), dehydroepiandrosterone (DHEA), androstenedione, testosterone and dihydrotestosterone. Testosterone is the most potent androgen, while DHEA-S, DHEA and androstenedione is considered to be androgen precursors that require further conversion to testosterone, either in peripheral tissue or in the ovary, to acquire an androgenic effect. Dihydrotestosterone is a peripheral product of testosterone that cannot be aromatized to estradiol (48). In PCOS, high circulating levels of androgens, estrogens, sex steroid precursors, and glucuronidated androgen metabolites have been demonstrated by gas chromatography tandem mass spectrometry (GC-MS/MS) and liquid chromatography tandem mass spectrometry (LC-MS/MS)(49). The major androgen excess in PCOS originates from the ovaries, but the adrenals contribute to some part (50). Hyperinsulinemia, often found in PCOS, inhibit the production of sex hormone binding globulin (SHBG), and thereby further contribute to increased levels of free circulating androgens (51).

Neuroendocrine dysfunction

Basic neuroendocrine control of follicle development

Follicle development and concomitant ovulation is under tight control by the hypothalamic-pituitary-ovarian axis. Gonadotropin releasing hormone (GnRH) neurons dispersed in hypothalamus project into median eminence where they release GnRH in a rhythmic pulsatile nature into pituitary portal circulation (52-56). The exact timing and control of GnRH secretion is regulated via an interplay of a network of different hypothalamic nuclei (57). As a direct result, GnRH, determines episodic/pulsatile secretion of gonadotropins; luteinizing hormone (LH) and follicle stimulating hormone (FSH) from the anterior pituitary into peripheral circulation

where they reach and affect the ovaries (58, 59). The periodicity and amplitude of GnRH and gonadotropin secretion is crucial for the entire reproductive axis and vary throughout the menstrual cycle (60). Slow GnRH pulses favor FSH while high frequency favors LH secretion (61-63). During the follicular phase GnRH gradually increase and force a LH surge that triggers ovulation. Following ovulation, a lowering of GnRH instead promotes FSH secretion (61). The neuroendocrine secretion is in turn controlled by the ovarian steroid production via feed-back mechanisms (64). Progesterone, a part of the ovarian sex steroid feedback system, is the main modulator of GnRH secretion (65) while estradiol plays a more permissive role with effects on pituitary progesterone receptors (66), whereas little is known about any possible androgenic modulatory effect.

Neuroendocrine disturbances in PCOS

In PCOS the disturbed hypothalamic-pituitary-ovarian (HPO) axis has been extensively reviewed (67-70). The most evident neuroendocrine feature regulating abnormal ovarian follicle development in PCOS is increased LH pulsatility regarding both frequency and amplitude, with relatively low FSH secretion (71-75). Increased LH pulse frequency increases theca cell production of androgens while the lower FSH levels impairs follicle maturation and consequently ovulation (68).

The cause of LH hypersecretion in PCOS is probably due to enhanced pituitary sensitivity to GnRH, or to changes in GnRH secretion patterns rather than increased GnRH secretion (71, 75, 76). It appears to be a result of an acquired impaired sensitivity of the hypothalamic pulse generator to the negative feedback of estrogen and progesterone in PCOS, possibly by chronic estrogen exposure (71, 74, 75, 77). Levels of FSH in PCOS appears to be low or within the lower follicular range and response to GnRH is relatively similar to ovulatory controls (71). There are several hypothesis for explaining this, firstly that increased GnRH frequency would favor LH secretion, secondly that the inhibitory action of estrogen is preferential for FSH suppression in comparison to LH, and thirdly that FSH secretion is not as sensitive to GnRH stimulation as LH (71, 78). Moreover, although LH increases are coincident with GnRH increases, this is not entirely the case with FSH and there is evidence of an additional control system. There is several other factor(s) that could affect FSH release with gonadal steroids as well as both gonadal and pituitary produced activins, inhibins, and follistatins as possible actors. Moreover, there is evidence to support existence of a separate FSH releasing factor although it has not been isolated (78). Altered sex steroid production, metabolic dysfunctions and obesity may all contribute

to the changes in LH secretion pattern. Hyperandrogenemia itself may cause hypothalamic desensitization to progesterone/estrogen negative feedback signals that further increase gonadotropin secretion and hence ovarian androgen production, causing a self-driven viscous circle (79, 80). Although increased BMI has a blunting effect on LH secretion (72, 75), hyperinsulinemia and insulin resistance may directly or indirectly (by enhancing ovarian gonadotropin stimulated sex steroid production) contribute to the abnormal gonadotropin secretion, although the mechanism is not clear (81-83). All these factors increase free androgen levels and contribute to anovulation.

Ovarian dysfunction

The ovarian dysfunction of PCOS involve both the morphological features of polycystic ovaries, with an accumulation of small antral follicles of size 2-9 mm, and the clinical consequence of oligo-/anovulation. Moreover there are follicle abnormalities where the most consistent feature is androgen hypersecretion (84).

Abnormalities in ovarian steroidogenesis

Ovarian steroid production is based on a close collaboration between theca and granulosa cells in the growing follicles, and requires gonadotropin input (figure 1) (85). Theca cells differentiate around growing follicles and immediately start to respond to LH by producing androstenedione from cholesterol, either by the Δ4 or Δ5 pathway. The ability to convert androstenedione to estrone and thereafter estradiol is exclusively acknowledged aromatase cytochrome P450 hydroxylase (CYP19) containing granulosa cells, and is under the control of FSH (86). Ovarian originating testosterone, which corresponds to about 75% of the circulating pool, is converted from androstenedione by 17β-hydroxysteroid dehydrogenase (17β -HSD) type V that mainly is expressed in the theca cells (87). Other isoforms of 17β –HSD, type I and II, are found in granulosa cells but is believed to be more associated to the conversions between estrone to estradiol (88). The circulating testosterone therefore mostly originates from theca cells.

LC-MS/MS measurements of follicular fluid of PCOS women have shown increased levels of both individual and total pool of androgens (DHEA, androstenedione, testosterone, androstanedione) and lower levels of individual and total pool of estrogens (estrone, estradiol, estriol) (89). Their increased production of testosterone

and androstenedione seems to be mainly via the Δ5 pathway and DHEA, although data is scarce (90).

Δ4 pathway   Δ5 pathway

cholesterol
↓ CYP11A
pregnenolone
3β-HSD ↙   ↘ CYP17
Progesterone   17-OH pregnenolone
↓ CYP17   ↓ CYP17
17-OH progesterone   DHEA
CYP17 ↘   ↙ 3β-HSD
Androstenedione
↓ 17β-HSD
Testosterone

Androstenedione → CYP19 → Estrone
↕ 17β-HSD   ↕ 17β-HSD
Testosterone   → CYP19 → Estradiol

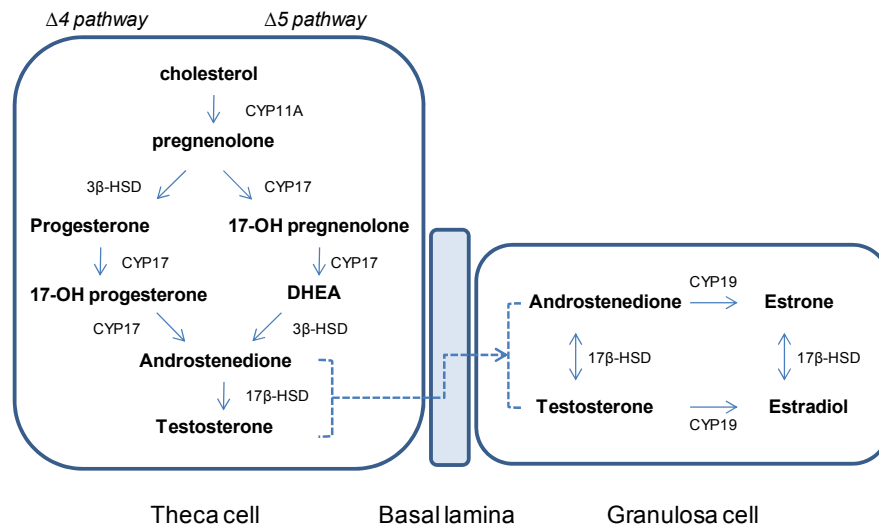Theca cell   Basal lamina   Granulosa cell

Figure 1. Steroid synthesis in the ovary

Women with PCOS appear to have theca interna hyperplasia, a thicker layer of the theca cells, which seem to be responsible for their increased androgen steroidogenesis. Moreover, each theca cell has increased expression of LH receptors, with increased susceptibility to LH stimulation (69, 86, 91). But there are also evidence supporting that hypersecretion of LH is not the primary cause of the abnormal steroid production in theca cells of PCOS ovaries, instead other intrinsic factors are primary responsible (92, 93). Expression and activity/efficacy of key enzymes involved in the steroidogenesis in theca cells has been shown to be increased in PCOS, with some support of a genetic origin (89, 91, 94, 95). Altogether, both number of androgen producing cells as well as their intrinsic and gonadotropin stimulated activity is increased in PCOS.

In granulosa cells the conversion of androgens to estrogens is dependent of FSH induced aromatase activity, which is increased in preovulatory follicles compared with non-ovulatory follicles (85). In PCOS, even though they have arrested follicular development, their steroidogenesis is active with increased aromatase activity and progesterone production compared with follicles of similar size from ovulatory women with or without polycystic ovaries (96). Same applies to the estradiol

production, which is typically increased in true anovulatory PCOS women with hyperandrogenism, but not in ovulatory subjects with polycystic ovaries (96-98). This creates a dividing line dependent on ovulatory status, where anovulatory subject have both hypersecretion of androgens and estrogens, while ovulatory subjects have solely hypersecretion of androgens. Moreover, testosterone itself has been shown to have a direct stimulatory effect on aromatase expression which could provide an explanation to this effect (99).

Hyperinsulinemia, one of the main features in PCOS, seems to trigger the thecal abnormalities and amplify ovarian steroidogenesis in both theca and granulosa cells (83, 100, 101). Reducing insulin secretion by metformin has been shown to decrease enzyme activity and circulating free testosterone while increasing SHBG levels (102). In lean women with normal insulin sensitivity reducing insulin levels was associated with decreased androgen levels, which also suggest increased insulin sensitivity in the androgenic pathway within the ovary (103).

Impaired follicle development

PCOS is a common reason for infertility and explain the largest part of WHO-II anovulation (104). The ovulatory dysfunction in PCOS can be ascribed the disturbed follicular development of excessive early follicular growth and abnormal later stages with arrested follicle growth well before expected maturation (105). This pattern of follicular growth with failure in the selection of a dominant follicle for ovulation, results in one of the hallmarks of PCOS, the PCO morphology.

PCOS ovaries were during the 80s found to contain 2-3 times as many small (2-5 mm) growing preantral follicles (106). This was later repeated where biopsies of both ovulatory and anovulatory PCO had increased density of small preantral follicles (107). Although there is an increased amount of growing follicles, it does not seem to cause premature depletion of the follicle pool. This may be explained by an concurrent and deviant decreased level of atresia of preantral follicles seen in PCOS (108). The arrested follicle development could be explained by their low FSH levels, not reaching the threshold needed to stimulate a normal maturation process (84). In addition, the hypersecretion of LH might suppress FSH function and cause premature luteinization of granulosa cells that is detrimental for follicle development and ovulation (109, 110). Granulosa cells from anovulatory PCOS has been shown to be significantly more responsive to LH than size matched control follicles which indicate prematurity (98).

Both androgens and insulin probably play a role in the impaired follicle development in PCOS. Anovulatory and ovulatory PCOS can be discriminated by insulin resistance and hyperinsulinemia, where these are more common features of anovulatory PCOS. Insulin may therefore contribute to the follicle dysfunction in PCOS, but it is probably not the only factor (96). Androgens impair follicle development by contributing to an exaggerated follicular growth at the early gonadotropin-independent stage. At later stages they work synergistically with LH and insulin by inhibiting granulosa cell proliferation or disrupting estrogen and progesterone synthesis, hence impeding follicular maturation (50).

There are also local selectors for follicle recruitment and growth within the ovary that might contribute to their impaired follicle development. AMH is expressed by early antral and preantral follicles, but not in later stages of development, and reflects the size and activity of the follicular pool (111, 112). There is also evidence of its involvement in the regulation of recruitment of primordial follicles into the growing pool, presumably by decreasing the granulosa cell sensitivity to FSH (113). Adding AMH to ovarian cultures reduces the number of growing follicles, while if removing the gene the proportion increases (114, 115). In the small primordial and transitional follicles of anovulatory PCOS, AMH protein expression is reported to be reduced (116). This may contribute to the inappropriate recruitment of growing follicles. Additionally in both circulation and antral follicular fluid of PCOS women, AMH levels are increased and these are associated with poor reproductive responsiveness to treatment (117-123). These high circulating levels may only be a reflection of their increased pool of granulosa cells in the follicle and not increased expression. Since high levels of AMH are associated with lower levels of FSH and estradiol it has been suggested that the AMH excess is involved in the lack of FSH-induced aromatase activity that is characteristic of follicular arrest in PCOS (122, 124). Testosterone exposure down-regulates AMH expression in granulosa cells of small bovine follicles in culture and could possibly represent a mechanistic origin of PCOS (125).

Ovarian inhibins are expressed in ovaries and acts as modulators to suppress FSH levels. As a response to increased FSH levels inhibin B is increasingly expressed during early follicular phase in small developing follicles, while inhibin A is selectively produced in the dominant follicle. Inhibin B therefore correlates to total follicle number and may be a marker of follicle quality (126). But inhibins also have a local effect and stimulate androgen production synthesis in theca cells for estradiol production (126-128). In PCOS most studies does not find any basal differences in circulating inhibin B, but an abnormal and increased response to FSH. The normal

basal levels may be explained by the diminished FSH secretion in PCOS or that the follicle quality is lower and the increased response to FSH could simply be due to the increased number of preantral and small antral follicles (127-130). Moreover, follicular arrest in PCOS is associated with reduced levels of both inhibin A and B in follicular fluid, which both could explain the normal circulating levels even though the increased pool follicles, but also making these possible actors in the impaired follicle development (131). There is low evidence of any diagnostic value of circulating basal inhibin B measurements in PCOS (127, 132).

Taken together, impaired folliculogenesis and steroidogenesis in PCOS seems to be multifactorial and is probably influenced by extra ovarian factors such as androgens, insulin, neuroendocrine alterations and intraovarian local and intrinsic factors as well as genetics.

Adrenal dysfunction

The adrenal cortex is the final and crucial part of the hypothalamus-pituitary-adrenal axis. It secretes both cortisol and androgens, such as DHEA, DHEA-S, androstenediol, testosterone and 11β-hydroxyandrostenedione, as a response to pituitary ACTH after hypothalamic corticotrophin releasing hormone (CRH) release (133). PCOS appear to have an increased adrenocortical activity resulting in increased levels of adrenal androgens with a relation to altered cortisol metabolism (134-137).

Adrenals contribute to PCOS hyperandrogenemia

Although the main part of androgen excess in PCOS origins from the ovaries 20-50% of patients also have an adrenal hyperandrogenism. DHEA-S, the sulfated form of DHEA, has clinically been the marker of adrenal androgen excess since 97-99% of the circulating levels origins from the adrenals as compared with testosterone where only around 25% origins from the adrenals. However, it may not always reflect the adrenocortical secretion of other adrenal products, either basally or as a response to ACTH (138). Serum levels of ACTH appears to be normal and, although the mechanism is not completely clear, data favors that the adrenal androgen excess in PCOS is more related to adrenal hyper responsiveness to ACTH than pituitary hyper responsiveness to CRH (139, 140). Altered enzymatic activity of cytochrome P450 17α-hydroxylase (CYP17) has also been reported and been suggested as a contributing factor to the adrenal hyperandrogenism (140, 141).

Cortisol

Glucocorticoids redistribute adiposity to central depots, increase the size and number of fat cells and may play a role in the development of metabolic syndrome (142). Cortisol is released in bursts from the adrenal gland with amplitude modulation rather than frequency modulation to control the nyctohemeral rhythm (143). Even though there is an increased ACTH response to CRH in obesity, the cortisol response seems unaltered. Instead more evidence point towards an altered peripheral cortisol metabolism with tissue specific cortisol excess (144-146). In PCOS plasma levels of ACTH and the response to CRH seems to be normal, suggesting normal pituitary responsiveness (138). Some, but not all data, indicate increased cortisol levels, both basally and ACTH stimulated, in PCOS (136, 147-149). However, there are evidence for increased peripheral cortisol metabolism and increased clearance from blood (134, 150-152). There has been focus on the activity of the enzyme 5α-reductase, which was found to be increased in both lean and obese PCOS and could explain part of the increased levels of cortisol metabolites (134, 151). Increased 5α-reductase has also been shown to be correlated to both HOMA-IR and BMI, indicating that insulin enhances 5α-reduction (151). Moreover, treatment of obese Zucker rats with the thiazolidinedione rosiglitazone, an insulin sensitizer and PPARγ agonist, significantly decreased 5α-reductase (153). One hypothesis suggests that the increased rate of cortisol metabolism *per se* would decrease the negative feedback on the HPA axis to compensate for the reduced levels of cortisol by increasing the adrenal drive and thereby also the androgen production. However, recent studies showing similar cortisol half-lives and similar pioglitazone- (another thiazolidinedione and insulin sensitizer) -induced reduction in 5α-reductase activity without significant effects on adrenal androgen or cortisol secretion, does not support this hypothesis (148, 154).

Metabolic disturbances

Insulin resistance with compensatory hyperinsulinemia is almost, but not quite, a universal feature of PCOS with prevalence between 65-80% in lean and up to 95% in obese subjects (155, 156). It is worsened by obesity since the obese PCOS population also add on the burden of insulin resistance that is related to excess adiposity (157, 158). Lean women with Rotterdam diagnosed PCOS have been found to be equally insulin resistant as obese controls while the insulin resistance of obese women with PCOS were even worse than the obese control group. Also, BMI had a more potent negative impact on insulin resistance in PCOS than in controls. Altogether, PCOS

have both an worsened intrinsic and extrinsic (BMI inflicted) insulin resistance (159). The metabolic disturbances in PCOS seem to be foremost inflicted on the classic hyperandrogenemic phenotypes of PCOS, whilst the anovulatory women with normal androgen levels are metabolically relatively healthy (8, 160-162). But it is not established whether the hyperinsulinemia related to insulin resistance causes hyperandrogenism or *vice versa* (42).

The metabolic syndrome is a constellation of multiple risk factors that consist of atherogenic dyslipidemia, elevated blood pressure, elevated plasma blood glucose, central obesity, a protrombotic state, and a proinflammatory state. The metabolic syndrome is associated with a ≈2-fold increased risk of cardiovascular events and a ≈5-fold risk for developing diabetes and the underlying risk factors are abdominal obesity and insulin resistance (163). The common features of obesity and insulin resistance in PCOS should subsequently result in increased prevalence of metabolic syndrome. Studies are inconsistent with varying prevalence, although most showing increased prevalence of metabolic syndrome with positive correlations to weight, age, hyperandrogenemia and low SHBG (3, 4, 164-167). These variations may be related to the studied population, PCOS definition, age and BMI.

PCOS is also related to high prevalence of impaired glucose tolerance and type 2 diabetes, with an accelerated development of type 2 diabetes from impaired glucose tolerance (167-169). Altogether, if above mentioned risk factors are not properly handled they will potentially lead to cardiovascular disease (CVD) later in life (170). Although increased prevalence of several risk factors, including increased serum concentration of CVD risk biomarkers, are found in PCOS women, hard evidence for increased CVD morbidity and mortality is inconclusive and long-term prospective studies are needed (1, 10, 11, 171, 172).

Basic insulin signaling and glucose transport

Insulin dependent glucose transport and metabolism in skeletal muscle and adipose tissue are regulated via the insulin-signaling pathway in which the glucose transporter 4 (GLUT4) is translocated into the plasma membrane to enable glucose influx from the blood. Signal transduction is mediated via a complex network of phosphorylation cascades after an initial activation of the insulin receptor substrate 1 (IRS-1) when insulin is docking the insulin receptor (173, 174) and is visualized in Figure 2. IRS-1 then activates phosphatidylinositol 3-kinase (PI3K) that further phosphorylates membrane phosphatidylinositol-4,5-bisphosphate (PIP2) to phosphatidylinositol-

3,4,5-triphosphate (PIP3). These activates phosphoinositide-dependent protein kinases (PDK-1 and 2) which can activate protein kinase B (Akt) that further phosphorylates Akt substrate of 160 kDa (AS160), and atypical protein kinase C λ and ζ (PKC λ/ ζ). These lastly stimulate GLUT4 translocation to the cell membrane (42).
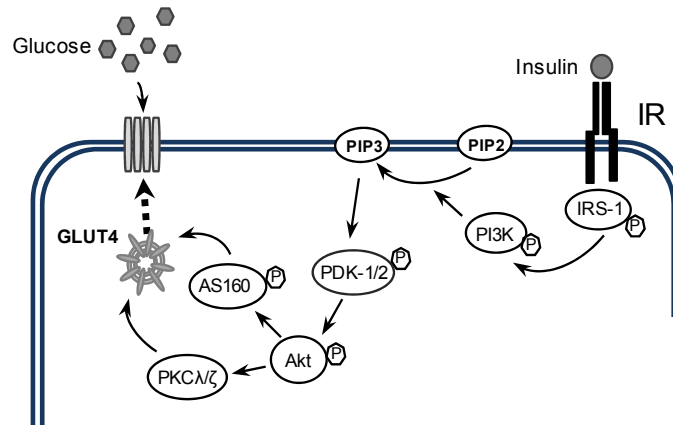

Figure 2. Signaling pathway resulting in the translocation of the glucose transporter GLUT4 in response to insulin. Akt: protein kinase B , AS160 – akt substrate of 160 kDa, IR – insulin receptor, IRS-1 – insulin receptor substrate 1, GLUT4 – Glucose transporter 4, PDK – phosphoinositide-dependent protein kinase, PI3K – phosphatidylinositol 3-kinase, PKC – atypical protein kinase C, PIP2 – phosphatidylinositol-4,5-bisphosphate, PIP3 – phosphatidylinositol-3,4,5-bisphosphate.

Basic insulin independent glucose transport

Glucose transport in skeletal muscle is regulated by at least two distinct signaling pathways that comprise the insulin stimulated signaling and the contraction/exercise insulin independent signaling (175-177). Both acute and chronic exercise training/contractions stimulate GLUT4 translocation, glucose uptake and insulin sensitivity (175-181).

The effect of acute contractions on glucose uptake is insulin independent with an additive effect to insulin stimulation and probably involves several different mechanisms (179, 180, 182). Acute exercise has also been shown to increase GLUT4 translocation by recruitment from a different intracellular pool than insulin stimulation, further indicating different mechanisms (182, 183). One mechanism may involve an interaction with distal insulin signaling by a convergence of different signaling branches. Although the converging link between the insulin signaling

pathway and insulin independent pathway has not been shown it has been speculated to be AS160 (177, 184). Other likely candidates involved in the contraction response of increased glucose uptake are cytosolic Ca^{2+}, 5' adenosine monophosphate-activated protein kinase (AMPK), and adiponectin (175, 176, 185, 186).

Repeated exercise training may restore protein signal transduction earlier in the insulin signaling pathway as proximal as PI3K and possibly also IRS-1 and 2 although data regarding IRS proteins are scanty (181, 187). MAPK's and/or AMPK may be involved in the cellular adaptations to chronic exercise with regards to increased gene expression (175-177, 181, 186).

Insulin resistance in PCOS

In general, insulin resistance can be defined as when a normal amount of insulin produce a less than normal biological response. This includes reduced responsiveness, reduced sensitivity, or both. In reduced responsiveness the maximal response to insulin is reduced, whereas in reduced sensitivity the acquired amount of insulin needed to obtain a certain response in increased (188). Sensitivity is usually explained by receptor binding or phosphorylation, whilst responsiveness is explained by post-receptor events (189).

Whole body insulin resistance is normally characterized by increased circulating levels of insulin, both basally and after a glucose load (190). The euglycemic hyperinsulinemic clamp is the golden standard method to assess insulin resistance, but it is impractical in clinic and oral glucose tolerance tests (OGTTs) or HOMA-ir indexes are instead common. But even though a normal result is obtained by an OGTT, insulin resistance could be concluded by HOMA-ir or clamp assessments (191, 192). Hence, to determine and diagnose insulin resistance, one single method may not be sufficient.

Muscle tissue corresponds to the uppermost part of whole body insulin stimulated glucose uptake, while adipose tissue only accounts for a small fraction (193). However, this does not mean that adipose tissue is not involved in whole body insulin sensitivity. On the contrary, adipose tissue seems to indirectly induce insulin resistance in other insulin target tissues through cross-talk mechanisms (194). Particularly visceral obesity causes insulin resistance that appears to be related to lipid accumulation in liver and induction of inflammation (195). In both lean and obese women with PCOS whole body insulin sensitivity (half-maximal insulin response) and responsiveness (maximum insulin response) has been shown to be decreased (190). It

has been suggested that obese women with PCOS in addition have decreased hepatic insulin sensitivity for endogenous glucose suppression, although this was not supported by later studies using updated tracer techniques (196, 197). With decreased insulin sensitivity, pancreatic insulin production increases, and an acquired pancreatic β-cell dysfunction is required for later development of type 2 diabetes. PCOS women display β-cell dysfunction independent of obesity, but more pronounced if having a first-degree relative with type 2 diabetes (190). Moreover, the size of subcutaneous adipocytes has been shown to be increased and serum adiponectin decreased in women with PCOS. Adipocyte size together with adiponectin and waist circumference was also strongly associated to insulin sensitivity (20).

Insulin resistance in muscle

Insulin resistance in muscle is defined as impaired glucose transport and muscle glycogen synthesis in response to insulin (195). Ciaraldi et al found that glucose transport in cultured myocytes originating from PCOS women have impaired insulin responsiveness but not sensitivity (158). However, no differences in GLUT4 levels have been reported (158, 171). Courbould's in vivo experiments of skeletal muscle biopsies during a clamp have shown a decreased IRS-1 associated PI3K activity, independent of obesity, along with decreased insulin mediated glucose disposal. This change was seen only early in the clamp. No change in insulin receptor (IR), IRS-1 or the p85 subunit of PI3K abundance was discovered, which is indicative of a defect in downstream signaling patterns. Levels of IRS-2 were though increased, which might reflect a compensatory effect (198). Following the insulin signaling pathway further downstream, another study found reduced levels of insulin stimulated phosphorylation (activation) of Akt and AS160 after a clamp with physiological doses of insulin, mainly measuring insulin sensitivity. They did not find decreased IRS-1 associated PI3K activity, which is consistent with the Courbould study, since here they took their biopsies after 3h of insulin infusion (199). This was not supported by the later Ciaraldi study who found no alterations in Akt activation (200). The reason may be that they used maximal insulin stimulation which might have masked this difference. To study whether the defects in skeletal muscle of PCOS is an intrinsic effect or environmentally induced, differentiation of myoblast into myotubes and then culturing them for generations has been implemented. Insulin resistance seems to be an acquired feature but some of the abnormalities, such as increased inhibitory phosphorylation of IRS-1 and an increase of IRS-1 abundance, that might have been a compensatory effect, may be intrinsic (201, 202).

Insulin resistance in adipose tissue

Insulin resistance in adipose tissue is defined as impaired glucose transport and inhibition of lipolysis in response to insulin (195). One consistent feature of PCOS adipocytes is decreased insulin sensitivity (191, 196, 200), indicating altered receptor binding or phosphorylation (170). IR number and insulin binding appears though to be normal, although the levels of the IR transmembrane β-subunit have been reported to be lower in one study (196, 203, 204). Some studies have found reduced insulin responsiveness indicating also post-receptor alterations (165, 205) that probably is related to the reduced levels of GLUT4 in PCOS adipocytes (204, 205). This data has been challenged and was contradicted by a study that did not find lower levels of GLUT4 in PCOS adipocytes (158). In isolated adipocytes no major differences in expression or activity of proteins downstream of IR in the insulin signaling pathway has been found. But in adipose tissue increased levels of PI3K together with impaired phosphorylation pattern of IRS-1 have been reported (158, 189). The drawbacks of these studies are that measurements were made after maximal insulin stimulation (responsiveness) and in basal state, respectively. This means that neither of them really represents, or explains, the most prominent feature of decreased sensitivity.

Increased sympathetic activity

The autonomic nervous system consists of two divisions; the sympathetic and the parasympathetic nervous systems, and is controlled by the neurotransmitters noradrenaline and adrenaline and activation of adrenergic receptors. In a normal, healthy state, a fine balance between the two divisions ensures homeostasis. Many of the classical components of PCOS such as polycystic ovaries, insulin resistance with related hyperinsulinemia, central obesity and hypertension are associated with increased sympathetic activity (206-209). It has therefore been suggested to account for at least a part of the syndromes etiology (207, 208, 210). That increased sympathetic innervation of the ovaries might contribute to the impaired follicular development in PCOS is supported by clinical evidence such as increased density catecholaminergic nerve fibers, increased NGF production and altered catecholamine metabolism and/or uptake in PCOS ovaries (208, 211-213). Heart-rate recovery after a bout of exercise and heart-rate variability can be used as non-invasive markers of autonomic function. Measures in PCOS women indicate that they have decreased dynamic activity in their autonomic function, possibly by decreased activity in the parasympathetic component and increased in the sympathetic component (214-216).

These are though indirect measures and their accuracy may be questionable. We have though demonstrated by microneurography (MSNA), which is a direct and reliable measure of muscle sympathetic nerve activity, that PCOS women have an increased sympathetic nerve activity that is correlated to high levels of testosterone (217).

PCOS – a well orchestrated pathology

Androgens play a central part, perhaps even the leading part, in the pathology of PCOS. Androgens alone can affect many of the systems that are impaired in the syndrome, and are sufficient to cause PCOS like states in both animal models and female-to-male transsexuals (27, 28, 30-32, 218-220). But these alterations can themselves further increase hyperandrogenemia. Consequently a vicious circle is created where the individual pieces may augment each other, and it is not clear where it started. In this section I will try to merge these pieces, similar to a "connect the dots" puzzle, illustrated in fig. 3. Firstly, I would like to point out that the effect of androgens can be mediated either directly via the androgen receptor, or through the aromatization to estrogen.

Hyperandrogenemia in PCOS originating mainly from the ovaries and can have central effects by increasing gonadotropin secretion via effecting sex-steroid feed-back systems as well as enhancing the effect on ovarian gonadotropin stimulated sex steroid production (50, 79, 80). Androgens also directly impair follicle development and maturation and thereby contributing to the PCO morphology and thereby the ovarian pool of androgen producing cells (50). Both of these will further drive ovarian androgen production and increase levels of free circulating androgens. Additionally, although the mechanism is not completely clear, increased adrenal androgen production contribute to the androgen excess in PCOS (139, 140).

Androgens are also associated with an atherogenic blood lipid profile, enlarged adipocyte size and peripheral insulin resistance although the androgen excess may not be the primary cause of their insulin resistance (20, 221, 222). Moreover, together with obesity this increases the risk of type 2 diabetes and CVD (15). Similar to androgens, insulin resistance and hyperinsulinemia enhances ovarian gonadotropin stimulated sex steroid production (50, 81-83) and may contribute to the abnormal gonadotropin secretion although the mechanism is not clear (81-83). Hyperinsulinemia also decrease liver production of SHBG which increases the amount of bioavailable free circulating sex steroids (51).

PCOS is related to increased muscle sympathetic activity and of special interest is that testosterone concentration was found to be a strong independent predictor (217). Increased sympathetic nerve activity is related to insulin resistance with related hyperinsulinemia, central obesity and hypertension (206-209) and might contribute to the increased cardiovascular risk (217). There is also evidence that support an increased sympathetic nerve activity to the ovaries (208, 211-213) that may further drive androgen production and PCO morphology (223). Apart from above described factors there is a strong genetic component with familial aggregation of cases and symptoms (43, 44), that probably is involved in the etiology of PCOS. Altogether, PCOS is a coordinated pathology containing factors that strongly influence each other, making it difficult to separate the etiology.
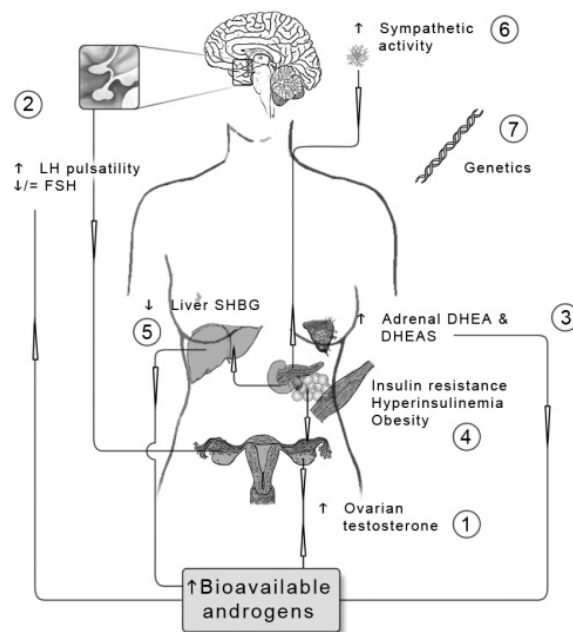

Figure 3. Summary of the PCOS pathophysiology. 1) Ovarian androgens are the main source of hyperandrogenemia in PCOS. Hyperandrogenemia have both a direct effect on the ovarian alterations as well as 2) an increasing effect on pituitary LH pulse frequency and amplitude with relative low FSH secretion. 3) Further, adrenal androgens contribute to PCOS androgen excess.4) Insulin resistance with compensatory hyperinsulinemia enhances ovarian androgen production as well as 5) decreasing production of SHBG in the liver, both increasing the pool of bioavailable androgens. 6) PCOS is also associated with increased muscle sympathetic nerve activity that is related to high testosterone, insulin resistance and obesity 7) Genetic defects probably contribute to the pathology of PCOS. LH – luteinizing hormone, FSH – follicle stimulating hormone, SHBG – sex hormone binding globulin, DHEA – Dehydroepiandrosterone, DHEAS – Dehydroepiandrosterone sulphate.

Treatment of PCOS

Due to the lack of knowledge about the etiology of PCOS and the heterogeneity of the syndrome no cure can currently be offered. Women are instead treated in a symptom oriented manner, often for long duration, with adverse effects.

Lifestyle modification

Lifestyle modifications, including diet and exercise, are frequently recommended as a first-line treatment in the large population of overweight and obese PCOS women. It improves several of the key features of PCOS such as body composition, hyperandrogenism and cardiometabolic profile, including insulin sensitivity and blood lipids, but also autonomic function and inflammatory pattern (224-233). The effect on reproductive function is though controversial and evidence from systematic reviews of randomized controlled studies (RCT's) is limited. However, it may improve ovulatory function and pregnancy (225, 227, 230, 234, 235) alone or together with clomiphene citrate (CC) (229). Even a modest (5%) weight reduction has been reported to improve metabolic and reproductive function (236). Although exercise training in overweight/obese PCOS women improves insulin sensitivity it does not seem to be able to normalize it to overweight control levels, making other treatment for IR sometimes still considered required (228). The effect of exercise on insulin sensitivity seems to be via mechanisms unrelated to weight loss (228) or mitochondrial function (224). Since skeletal muscles represent such large part of the body mass and might account for up to 90% of the insulin-stimulated uptake, most focus of the effect of exercise has been on muscle adaptations (237).

Pharmaceutical and surgical alternatives

For PCOS women not trying to conceive, combined oral contraceptives (COC) are commonly used for menstrual disorders associated with PCOS. COC reduce hyperandrogenism by suppressing pituitary LH hormone secretion and ovarian androgen secretion while increasing circulating SHBG. Consequentially, they improve signs of hyperandrogenemia in form of acne and hirsutism (238). Although limited evidence and lack of long-term effects, COC may have negative impact on metabolic function and insulin resistance, especially in obese PCOS patients (158, 238).

Insulin sensitizers

Insulin resistance and hyperinsulinemia have implications for both ovarian function and long-term health related to metabolic abnormalities. Considering to treat women with PCOS with insulin sensitizers are therefore common and logical (239).

Metformin, originally used world-wide for the treatment of type 2 diabetes, inhibits hepatic glucose output and increases insulin sensitivity and glucose uptake in peripheral tissues (240). It is now also commonly used in PCOS for improving insulin sensitivity and several studies show decreased insulin, androgen and LH levels, improved LH pulsatility by reduction of amplitude, cortisol secretion frequency and potential effects on reproductive function (81, 241-247). Adverse effects include gastrointestinal distress such as nausea, abdominal pain and diarrhea and malabsorbtion of vitamin B_{12} (241, 242). Although commonly used it is currently not licensed as treatment for PCOS (242).

Thiazolidinediones such as pioglitazone and rosiglitazone was initially used to treat type 2 diabetes and improve peripheral insulin sensitivity but has later also been used to treat PCOS. Similar to metformin they increase insulin sensitivity and they may decrease free testosterone and DHEA levels but without any effect on LH pulsatility. Since, by contrast to metformin, they tend to increase weight and cause serious adverse effects, they are not recommended for women trying to conceive, at risk for pregnancy or with PCOS (1, 197, 239, 248, 249).

Ovulation induction

Treatment of first choice for ovulation induction in women with PCOS attempting to conceive is clomiphene citrate (CC) (245). CC is a non-steroidal compound that resembles an estrogen, enabling it to block hypothalamic estrogen receptors and the estrogenic negative feedback and thereby induce a FSH and LH discharge from the pituitary leading to ovulation (250-252). Predictors of outcome are mainly obesity and hyperandrogenemia but also mean ovarian volume and cycle history (253). Studies have shown an ovulation rate of 60-85% and a pregnancy rate of 30-50% after six ovulatory cycles; if ovulation cannot occur at the highest dose of 150 mg/day they are designated as CC resistant (approx. 20%) (253, 254). Ovarian hyperstimulation, multiple gestations and spontaneous abortions belong to the adverse effects of CC stimulation, although rare (245, 250). The reports on the efficacy of metformin in combination or as alternative to CC in PCOS and reproductive function is inconclusive and the latest Cochrane report from 2012 concludes an association with

higher clinical pregnancy rate compared with placebo and in combination with clomiphene citrate versus clomiphene alone (255). However, there was no improvement on pregnancy outcome (live births) either used alone or in combination with clomiphene, in contrast with clomiphene alone (255, 256). A following systematic review and meta-analyses concluded that there is insufficient evidence to confirm the superiority of either treatment but that metformin is associated with more adverse effects (257).

If CC fails, gonadotropin (FSH) therapy or laparoscopic ovarian surgery is considered as further treatment. Low dose protocols of FSH are now recommended for PCOS to reduce the risk of excessive follicle development/multiple pregnancies (< 6%) and ovarian hyperstimulation syndrome (<1%) that were more frequent with traditional dose protocols. The efficacy manifests a 70% ovulation rate and a 20% pregnancy rate (245) but the treatment are costly and requires intensive ovarian monitoring (247).

Laparoscopic ovarian surgery (LOS) was initiated with ovarian wedge resection and has won ground with the rise of laparoscopy and other minimally invasive surgery such as laparoscopic ovarian drilling by electrocautery or laser. LOS has the advantage over gonadotropin therapy by lower risks of higher order multiples and ovarian hyperstimulation syndrome although a surgical option always include intra- and postoperative risks (247, 258, 259). The latest Cochrane review reported a pregnancy rate between 25-51% and live birth between 24-44% with no significant difference compared to CC or gonadotropins (260). LOS can be recommended for patients that hypersecrete LH since it reduces LH, LH/FSH, LH amplitude and pituitary responsiveness to GnRH in addition to reduction of androgens and estrogens, long term (261, 262). The mechanism behind LOS is yet unknown but speculations include destruction of androgen producing tissue and the peripheral conversion of androgens to estrogens (245, 260). Another possible mechanism may be modification of ovarian sympathetic nerve activity, since PCOS is associated with sympathetic overactivity and ovarian nerve growth factor (NGF) excess (207).

Acupuncture

Hypothetical mechanism

Acupuncture is widely practiced and is now also accepted in the western world for the treatment or adjunct treatment for more and more conditions (263). It is a relatively safe treatment with few side effects (264).

Acupuncture originates from Traditional Chinese Medicine where fine needles is placed in the skin and underlying muscle at specific areas of the body, so called acupoints. When placed, needles are stimulated manually, so called manual acupuncture, by rotating or perturbing needles up and down. Needles may also be stimulated electrically by applying an electrical field and passing an electrical current between two needles, so called electro-acupuncture (EA). Low-frequency (1-15Hz) electro-acupuncture with an intensity that evokes muscle contractions is believed to achieve biological processes that resemble the effects of exercise. Acupuncture from a western scientific perspective can't confirm point specificity and instead explain the effect with the activation of afferent sensory nerve fibers at appropriate segmental level (265, 266). Acupuncture activates and modulates nervous pathways at peripheral (local), segmental (in the spinal cord) and at supraspinal level within central nervous system (CNS).

Starting at a peripheral level, both manual and electrical stimulation increase glucose uptake and microcirculation (267-270). When needles are inserted and stimulated, peripheral nerve terminals releases several neuropeptides, such as neuropeptide Y (NPY), vasoactive intestinal polypeptide (VIP), substance P and calcitonin gene-related peptide (CGRP) an gives an immediate local reaction, by which the two latter is probably involved, with an increase in microcirculation (267, 271, 272). Moreover, data from administration of the opioid antagonist naloxone also suggest low-frequency EA stimulated peripheral opioid release (273).

Mechanoreceptors, responding to mechanic pressure or distortion, are activated by muscle contractions and are suggested to be involved in the somatic response of both manual and electrical stimulation (274, 275). Activation of mechanoreceptors by manual or electrical stimulation activates sensory nerve fibers; myelinated Aα, β, δ and unmyelinated C-fibers (276, 277). These signals are transmitted to the spinal cord (segmental level) where they through spinal reflexes may modulate the sympathetic output to the target organs in the same area of innervation as where there needles are

placed (278). Importantly, these signals are also controlled via supraspinal pathways within the CNS (279).

The efficacy of acupuncture for analgesia has been extensively studied and widely used. The involvement of endogenous opioids within the central nervous system has been suggested to mediate the effect of acupuncture-induced analgesia and lowering of blood pressure (280-283). There are three opioid receptors μ, δ and ϰ found in peripheral afferent nerve terminals and within the central nervous system in areas related to nociception and pain (284, 285). β-endorphin, one of the cleavage products of pro-opiomelanocortin (POMC), binds to the μ-receptor with high affinity and has received special attention (284). β-endorphin produced in the arcuate nucleus of the medial basal hypothalamus is released within CNS but it is also produced in the pituitary where it is released into the peripheral circulation (286-288). The β-endorphin produced in hypothalamus project to the midbrain (especially the peri-aqueductal grey) and brainstem nuclei where it can influence pain sensitivity and autonomic function (287, 288). The effect on autonomic function include effects on the vasomotor centre with regulation of blood pressure and muscle sympathetic activity (markers of sympathetic tone) (289). The other system is also under hypothalamic control, by corticotrophin releasing hormone (CRH), and involves the anterior pituitary lobe where POMC is cleaved into equal amounts of β-endorphin, melanocyte stimulating hormone and adrenocorticotrophic hormone (ACTH) that is released into circulation (290). The two systems work independently but both can be activated by afferent nerve activity such as manual and low-frequency electrical stimulation of acupuncture needles, and exercise (287).

The β-endorphinergic system is involved in many physiological effects, both centrally and peripherally, such as reproductive function, analgesia, stress response and carbohydrate metabolism (291). Both circulating and central levels of β-endorphin have been shown to be modulated by acupuncture (292-294). Further implications of acupuncture involving modulation of autonomic function are the reducing effect on muscle nerve sympathetic activity and blood pressure (295, 296). The pain relieving effect of acupuncture was further blocked by low doses of naloxone, an opioid receptor antagonist, and the blood pressure suppression by high doses of naloxone (296, 297).

The connection between central β-endorphin and reproductive function involves both direct and indirect tonic inhibitory effects on GnRH and subsequent LH release, and possibly also GnRH biosynthesis (288, 298). It is generally accepted that opioids mediate the inhibitory effect of estrogen (299). Moreover, a reduction in opioid

inhibitory tone amplifies, and is essential, for generation of the LH surge preceding ovulation (288, 300).

Circulating β-endorphin is considered to be more related to stress stimuli than actually reflecting central opioid activity and should not be used as a marker of central activity (301). Still, the changes in plasma β-endorphin provide a link to the HPA axis, since β-endorphin is co-released with ACTH from the pituitary (290). The controlling factor CRH is released by stress, but it is also known to decrease GnRH secretion, linking it back to the reproductive axis (302). Acupuncture has been shown to decrease levels of CRH within hypothalamus and may therefore present effects on both HPO and HPA axis (303).

In general the effect of acupuncture may also be influenced by the patient's own expectations, since there is a strong psychological component to it (304). The use of placebo in acupuncture experiments are therefore always under debate, but the use of so called placebo needles or minimal/sham acupuncture may not be recommended since clinical studies suggest they are not an inert treatment (305, 306).

Implications of acupuncture in PCOS

Since many of the features of PCOS is associated to disturbed opioid or sympathetic tone, including disturbed gonadotropin secretion, insulin resistance and central obesity (207, 291), they may be implicated in the pathogenesis of the condition. PCOS have higher levels of circulating β-endorphin which may contribute to the pathogenesis, possibly by insufficient inhibition of central β-endorphins on GnRH secretion (291, 307-309). This is supported by studies in which the μ-receptor antagonist naltrexone improves cyclicity and SHBG, reduces androgen levels, LH/FSH and LH response to GnRH (310-312). Naltrexone also results in improvements of metabolic parameters in PCOS indicating involvement of β-endorphins in insulin sensitivity (310, 312, 313). To study the therapeutic effects of acupuncture in PCOS may therefore be well-established, and is persistently performed in China although those data mostly are inaccessible due to language barriers.

The relation between acupuncture, β-endorphin and sympathetic activity seems to be sustained also in PCOS. Acupuncture treatment has been shown to reduce both high plasma β-endorphin and sympathetic nerve activity and increase low hand-skin temperature (217, 295, 314).

For ovulation induction, often the main outcome except for live birth when treating PCOS women, acupuncture has indicated beneficial effects. Case-control studies on acupuncture for women with PCOS as well as with undefined anovulation show improvements menstruation pattern, LH/FSH ratio, estrogen and testosterone with long lasting effects (314-316). The effect on the HPO axis was recently confirmed in our previous RCT in which acupuncture with combined manual and low-frequency (2Hz) electrical stimulation of needles was superior to exercise and no intervention in improving menstrual frequency *and* lowering circulating levels of testosterone in PCOS women (233).

Clinical data demonstrating metabolic responses are scarce, and even more so in PCOS although experimental data demonstrate improved insulin sensitivity by acupuncture in rat PCOS models (317). Recent reviews support that acupuncture may be beneficial for both obesity and insulin sensitivity in diabetes but the included clinical studies are underpowered with poor methodological quality and more well-performed RCT's are required (318, 319). There is evidence that points toward a relation between circulating β-endorphin and increased pancreatic insulin and glucagon secretion both experimental and in healthy, diabetes and PCOS subjects (320-323) implicating a hypothetically metabolic response of acupuncture in PCOS. A hypothetical mechanism for acupuncture in PCOS is presented in figure 4.
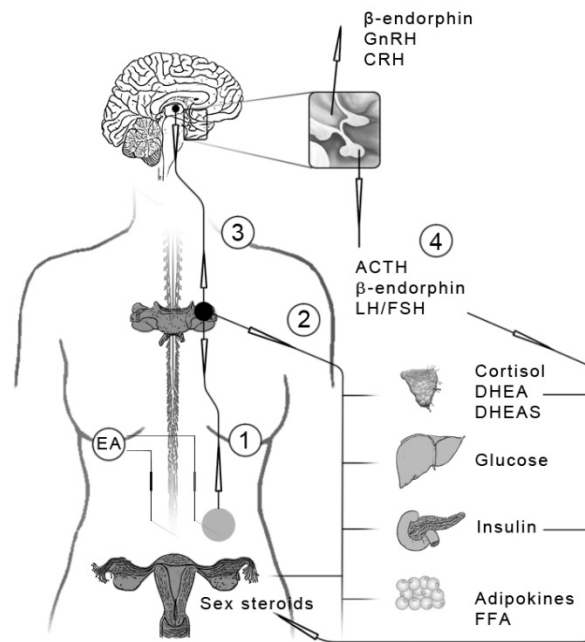
Figure 4. Schematic illustration of a hypothetical mechanism explaining the effects of acupuncture in PCOS. 1) Stimulation of acupuncture needles in skeletal muscle excites ergoreceptors that activate afferent sensory nerve fibers. These signals are transmitted to the spinal cord where they 2) through spinal reflexes may modulate the sympathetic output to the target organs in the same area of innervation. 3) Signals also reach the central nervous system via supraspinal pathways where they can exert central effects. Hypothalamic β-endorphin is implicated in the effect of acupuncture. It modulates the autonomic system but may also alter the release of GnRH and CRH. 4) These can enable an effect on reproductive function (via LH and FSH), adrenal function (ACTH), and pancreatic function (circulating β-endorphins). (ACTH – adrenocorticotrophic hormone, CNS – central nervous system, CRH – corticotrophin releasing hormone, EA – electro-acupuncture, FFA – free fatty acids, FSH – follicle stimulating hormone, GnRH – gonadotropin releasing hormone, LH – luteinizing hormone.)

Animal models of PCOS

Animal models are used to study etiology and pathophysiology, perform drug-screening and investigate effects of treatment in ways that are not possible in humans as well as to perform statutory pre-clinical studies. In order to mimic the pathophysiology of the syndrome several models in different mammalian and primate species have been developed during the years, each with its advantages and disadvantages. The unknown etiology of PCOS problematizes the use of these animal

models. Add on the difficulties to agree on diagnostic criteria as well as ethical considerations regarding use of animals for research and we can conclude that all of them involve limitations. They can help us understand some of the pathogenesis behind PCOS but they will not give us all the answers (29). The most informative animal models of PCOS are perhaps the prenatally androgenized sheep and rhesus monkeys that has offered knowledge of the role of androgens in fetal life in developing metabolic and reproductive abnormalities such as insulin resistance, anovulation and LH hyper secretion (29-32). Advantages over similar rodent models are that sheep and subhuman primates such as rhesus monkeys have a follicular development that is completed in utero, while rodents´ are completed postnatally (324). Although clinical manifestations in these sheep and rhesus monkey models are very similar to human PCOS the limitations of transcribing the results to human PCOS involve the uncertainty of common origin. Moreover, the achieved testosterone levels in these models are a great deal higher than in pregnant women with PCOS (325, 326). Since human and non-human primates placentas produce cytochrome P450 aromatase (CYP19a1) that converts androgens to estrogens, hence protecting the female fetus from high androgen levels, it is not clear how much of the maternal androgens that actually enter the fetus (327, 328).

Several rodent models of PCOS are currently described after exposure to androgens, estrogens, aromatase inhibitors and antiprogestins during intrauterine, prepubertal and adult life, but also from changes in light exposure and genetic mutations (39, 40, 226, 329-336). The reproductive and metabolic effects are inconclusive and seem to be dependent of timing and length of exposure, substrate and dose (218, 219). The rat model used in this thesis is based on prepubertal long-term and continuous administration of DHT to female rats, to mimic the pubertal onset of PCOS symptoms (24, 39) . It develops PCOS traits such as irregular cycles, polycystic ovaries as well as the metabolic features increased body fat, increased adipocytes size and insulin resistance (39). Benefits of rodent models are their short regeneration time and low costs. This makes it possible to study both reproductive status and transgenerational transfer of PCOS traits as well as long-term effects and treatment regimens. One must though consider the larger species differences in relation to humans given their rapid ovulatory cycle with multiovulatory pattern.

PRESENT INVESTIGATION

AIMS

General aims

The aim of this thesis was to evaluate the effect of acupuncture on ovulatory and neuroendocrine, as well as metabolic, dysfunction in the pathology of PCOS, and to search for potential molecular mechanisms mediating the effect.

Specific aims

Paper I   To investigate if continuous administration of DHT in female rats, starting before puberty, induces changes in the hypothalamic expression of AR, GnRH, GnRH-receptor, and CRH. To determine whether low frequency (2Hz) electro-acupuncture, with intensity high enough to evoke muscle twitches, restore estrus cyclicity and hypothalamic protein expression.

Paper II   To test the hypothesis that giving acupuncture with combined manual and low-frequency electrical stimulation to women with PCOS, increases ovulation frequency and improves LH pulsatility and sex steroid pattern to greater extent compared only to the attention part of the therapy.

Paper III   To test the hypothesis that low-frequency electro-acupuncture normalizes insulin sensitivity in rats with DHT-induced PCOS. To further investigate if this effect is achieved via restored molecular signaling related to insulin sensitivity and effects on lipid profile.

Paper IV   To test the hypothesis that manual- and low-frequency electrical stimulation of acupuncture needles improves whole-body insulin sensitivity in rats with DHT-induced PCOS, with electrical stimulation being more effective. To further investigate if the two treatment modalities have different effects on gene- and protein expression related to insulin sensitivity.

METHODOLOGICAL CONSIDERATIONS

The papers of this thesis are based on experimental data from three animal studies and one clinical study, where all four focus on PCOS and the effect of acupuncture. Experimental methods used have involved acupuncture treatment procedures, cyclicity-, insulin sensitivity- and body composition measurements, gene- and protein expression assessments and many, many biochemical analyses of blood samples.

Ethics

All experiments presented in this thesis have been approved by the Animal Ethics Committee (No. 23-2008 and 161-2010 [Paper I, III and IV]), or the regional ethical review board (No. 679-08 [Paper II]) of the University of Gothenburg, according to Swedish law. Care of animals was in agreement with the principles of the Guide to the Care and Use of Experimental Animals (www.sjv.se) and the human study was performed in accordance with the Declaration of Helsinki.

Animal studies

Animals used were Wistar rats from Charles River (Germany). They were housed at the Experimental Biomedicine facility, Sahlgrenska Academy, University of Gothenburg and kept under controlled conditions with 21–22°C, 55–65% humidity and a 12-h light, 12-h dark cycle. Rats were fed commercial chow and tap water ad libitum.

Animal model and study designs

Study designs of experiments of the animal experiments are presented in figure 5. The PCOS animal model used in studies I, III and IV was induced by subcutaneous implantation of 90-d continuous-releasing pellets containing 7.5 mg DHT (daily dose of 83 μg) into female rats. The pellets were implanted pre-pubertally (day 21) to mimic

the clinical manifestation of human PCOS around puberty/adolescence (24) and the dose was chosen to simulate the approximate 1.7 fold higher plasma DHT levels seen in PCOS women versus controls (337, 338). This dose has later been demonstrated to increase DHT in rats to supraphysiological levels, several fold higher than in control animals (253±21 pg/ml, vs. 31±3 pg/ml in controls), measured with radioimmunoassay (RIA) (339). However, the serum DHT levels in PCOS women might be underestimated and may not represent the actual concentration and androgenic bioactivity in the local tissues where DHT is converted from testosterone. The model display both the ovarian and metabolic characteristic of PCOS such as irregular cycles, polycystic ovaries, insulin resistance and obesity (39).
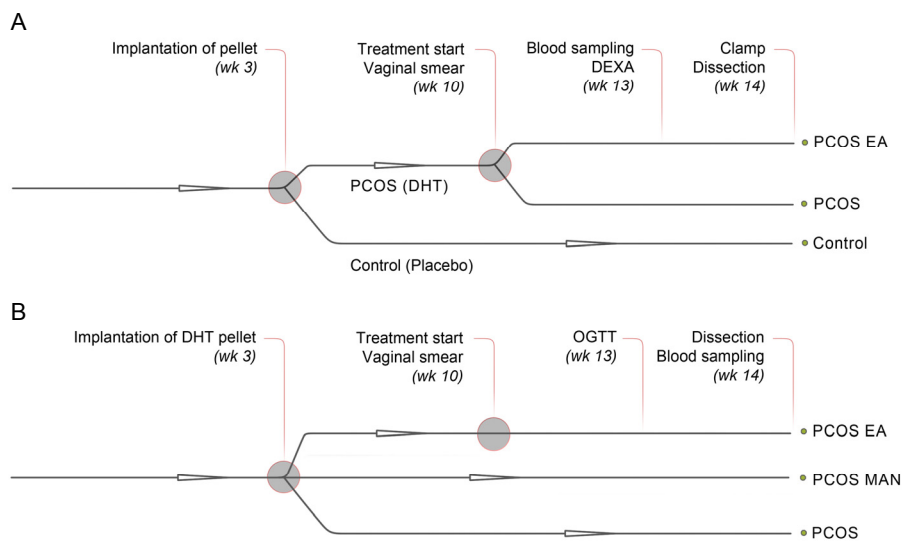

Figure 5. Study designs on time axes for experiment of A) paper III and B) paper IV. Pellets were implanted at wk 3 (21 d of age) while treatment began at wk 10 of age (7 wk after implantation of pellet). A) In paper III animals were divided in control (placebo pellet), untreated PCOS (DHT pellet) or PCOS receiving electro-acupuncture. Blood sampling was performed at wk 13 of age and the experiment was concluded with the clamp. B) In paper IV all rats received the DHT pellet and were either left untreated (control) or received manual acupuncture (PCOS MAN) or electro-acupuncture (PCOS EA). Rats that were not treated in the two experiments were still handled the same amount of time. DHT - dihydrotestosterone, wk – week, DEXA – dual-energy X-ray absorptiometry, EA – electro-acupuncture).

The experimental studies presented here were based on previous reports where we demonstrated that repeated acupuncture with low frequency (2Hz) electrical stimulation in female rats with DHT-induced PCOS increased low insulin sensitivity as well as improved cyclicity pattern and ovarian morphology (340). The aim of the present thesis was to elucidate the effect and mechanistic action of repeated acupuncture treatment. To be able to compare the results with previous studies, the same DHT-induced PCOS model was chosen with the same dose of DHT.

Treatment

The acupuncture with electrical stimulation treatment in paper I, III and IV was performed as previously described but with daily treatments (5 days per week) instead of three treatments per week (317). In brief, needles were inserted under light anesthesia in rectus abdominis and in triceps surae muscles, both bilaterally, in the somatic segments corresponding to innervations of the ovaries (T10-L2 and at sacral level). Animals were then awake during treatment session and needles were connected to an electrical stimulator and stimulated with low frequency (2 Hz) burst frequency. The intensity of stimulation was adjusted during each session to visible, non painful muscle contractions. To accustom the animals, length of each treatment was gradually increased from 15 min in the first week to 25 min the final week. Although animals were placed in fabric harnesses and suspended over the bench, they sometimes escaped the treatment. Animals were then re-anaesthetized and re-attached, but to a maximum of two times per day to avoid stress and excessive isoflurane impact. Rats were then treated as usual the following day, but were not excluded from the experiment. This escaping behavior was more frequent in the beginning of the treatment period but was not considered a problem in the end when the animals were used to treatment.

Clinical study

The clinical study described in paper II was conducted at the Sahlgrenska University Hospital, and at the Sahlgrenska Academy at University of Gothenburg, Sweden (341). It was registered at ClinicalTrials.gov: identifier NTC00921492 and is reported according to the CONSORT and STRICTA guidelines (342, 343).

Included subjects

PCOS patients were recruited by advertisement in local newspapers. Women were diagnosed according to the Rotterdam criteria and underwent two-dimensional transvaginal ultrasonography to examine ovarian morphology, and clinical examination of hyperandrogenism and menstrual frequency pattern. All participants gave informed oral and written consent before inclusion.

Inclusion criteria; fulfill two out of three:

- Polycystic ovaries (≥12 follicles 2–9 mm and/or ovarian volume ≥10 ml in one or both ovaries).

and/or

- Oligo/amenorrhea (oligoamenorrhea: intermenstrual interval > 35 days, less than six menstrual bleedings in the past year.

and/or

- Clinical signs of hyperandrogenism (hirsutism or acne). [Ferriman-Gallwey (FG) score ≥8 (344)| *Do you have excessive acne?* (Yes/no)].

Exclusion criteria

- <18 or >38 years of age.
- Body mass index (BMI) >30.
- Any pharmacological treatment within the last 3 months.
- Breastfeeding or received acupuncture within the last 24 weeks.
- Cardiovascular disease (CVD), diabetes mellitus, other endocrine disorders (e.g., congenital adrenal hyperplasia, Cushing´s syndrome or androgen-secreting tumors).

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Study design

The study design of the clinical study in paper II is presented in figure 6. Throughout the inclusion period patients were instructed to document menstrual bleeding with the aim to perform baseline assessments at day 8-12 in the cycle, and weekly progesterone measurements were performed to confirm ovulation. Computer-generated randomization to refer subjects to either acupuncture or attention control treatment was performed after baseline assessments to avoid influence by expectation of treatment. After the 10-13 weeks intervention period, with treatment twice per week, baseline assessments were repeated. Weekly progesterone measurements were performed throughout the intervention period.
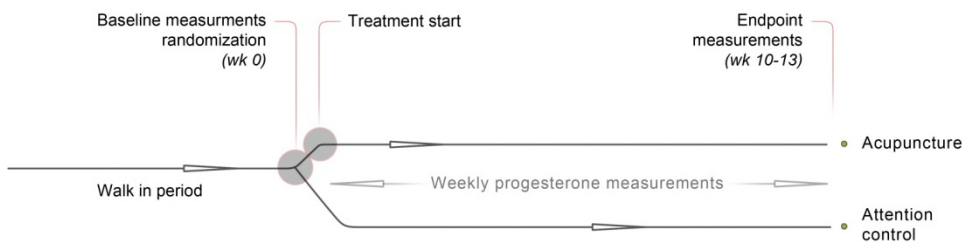

Figure 6. Study design on time axis for paper II. Randomization and allocation to either acupuncture or attention control intervention was performed immediately after baseline measurements. The treatment period, with weekly progesterone measurement to establish ovulation, lasted for 10-13 weeks and was concluded by repeating the baseline measurements.

Interventions

Both groups visited the same team of two physical therapists twice weekly, for 30 minutes per visit. Women were allowed to rest and listen to relaxing music during intervention. Women receiving acupuncture alternated between two sets of protocols to avoid soreness due to frequent treatments. The protocols were based on previous clinical (233) and experimental (279) studies, as well as clinical experience and are presented in Table 2.

Table 2. Used set of acupuncture points.

Points	Location	Stimulation	Innervation	Muscle location
Set 1				
CV3 CV6	Midline	EA, 2Hz	L1 Th11	Fibrous tissue, linea alba
ST29	Bilateral	EA, 2Hz	Th6-12	M. rectus abdominis
SP6 SP9	Bilateral	EA, 2Hz	L4-5, S1-2 S1-2	Mm. flexor digitorum longus, tibialis posterior M. gastrocnemius
LI4	Bilateral	Manual	C8, Th1	Mm. interosseus dorsalis I, lumbricalis II, adductor pollicis
GV20	Midline	Manual	C2-3	Aponeurosis epicranii
Set 2				
CV3 CV6	Midline	Manual	L1 Th11	Fibrous tissue, linea alba
ST25 ST29	Bilateral	EA, 2Hz	Th6-12	M. rectus abdominis
SP6 LR3	Bilateral	EA, 2Hz	L4-5, S1-2 S2-3	Mm. flexor digitorum longus, tibialis posterior M. interosseus dorsalis I
PC6	Bilateral	Manual	C8, Th1	M. flexor digitorum superficialis
GV20	Midline	Manual	C2-3	Aponeurosis epicranii

CV: conception vessel; EA: electro-acupuncture; GV: governor vessel; LI: large intestine; LR: liver; M: muscle; Mm: muscles; PC: pericardium; SP: spleen; ST: stomach.

Needles were placed in abdominal and leg muscles in the innervation area corresponding to sympathetic innervation of the ovaries and uterus. In addition, needles were placed in the hands. After insertion all needles were immediately stimulated manually by rotations to evoke *de qi* sensation. Thereafter needles in abdominal and leg muscles were stimulated electrically with low-frequency (2 Hz) burst frequency to induce muscle contractions, while remaining needles were stimulated manually, totally 4 times during treatment. To control for the attention involved in the treatment procedure, women in the attention control group were visited by the therapist, who ensured well-being, every 10 minutes during each stay. To avoid group differences in expectation of intervention, participants were thoroughly informed that it is not known whether the active component of the

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current intervention is acupuncture per se, and/or the attention of the therapist handling the needles. Women were also encouraged *not* to discuss the intervention with each other.

Sampling and outcome measures

During inclusion visit, patients underwent a trans-vaginal two-dimensional ultrasonography of the ovaries, measuring ovarian volume and number of antral follicles ≤ 9mm, as well as endometrial thickness. At the day of baseline assessments patients arrived after at least 4 hours of fasting (including food, coffee/tea/smoking). Anthropometric analyzes were performed before a standardized meal was provided. An intravenous venflon cannula was inserted 1 h before start of blood sampling to avoid stress interference. Blood sampling for LH and cortisol analyses were performed every 10 min for 12 h throughout the night. Blood sampling for FSH and sex steroid analyses were performed with 60 and 240 min intervals respectively. Encountered problems with blood sampling included difficulties with insertion and clotting of the venflon cannula. This could delay start of blood sampling or result in missing samples. Sleep quality during the night varied between subjects and should have been recorded and related to cortisol levels.

Estrous cyclicity and ovulation frequency

Estrous cyclicity (paper I, III-IV)

The estrous cycle in a rat is four days long and is divided into proestrus, estrus, metestrus and diestrus. Ovulation occurs between the beginning of proestrus and the end of estrus. Each stage is determined by the proportion of cell types present in the vaginal smears, i.e. epithelial cells, cornified cells and leukocytes. In proestrus the predominant cell type in the smear is nucleated epithelial cells; in estrus it is anucleated cornified cells; in metestrus it consists of similar proportions of leukocytes, cornified, and nucleated epithelial cells; and in diestrus the predominant cell type is leukocytes (Figure 7) (345). In paper I, III and IV analysis of vaginal smears were performed daily, from start of acupuncture treatment and throughout the study, to monitor the effect of acupuncture on the estrous cycle of the rats. Cycle stage was also determined to enable euthanization of cycling rats in the same stage of the cycle for correct comparison. Several physiological parameters, for example insulin sensitivity, are

known to fluctuate during the cycle in both rats and humans due to the impact of sex steroids. In the presented studies we performed clamp experiments and sacrificed animals during estrus. Since DHT rats are acyclic or have irregular cycles and display presence of leukocytes in their vaginal smears, indicating a *pseudo* diestrus (Figure 7) (39, 340), one may argue that a more correct comparison between groups would have been accomplished in diestrus stage. In rats, insulin sensitivity is at the lowest during estrus phase when estrogen is low and progesterone high (346, 347). Our rationale for choosing estrus is because of the low and relatively stable estradiol levels together with low insulin sensitivity (the main outcome measurement). This would ensure that potential differences in insulin sensitivity between groups were true, since PCOS rats were compared with control rats in their stage of lowest insulin sensitivity.
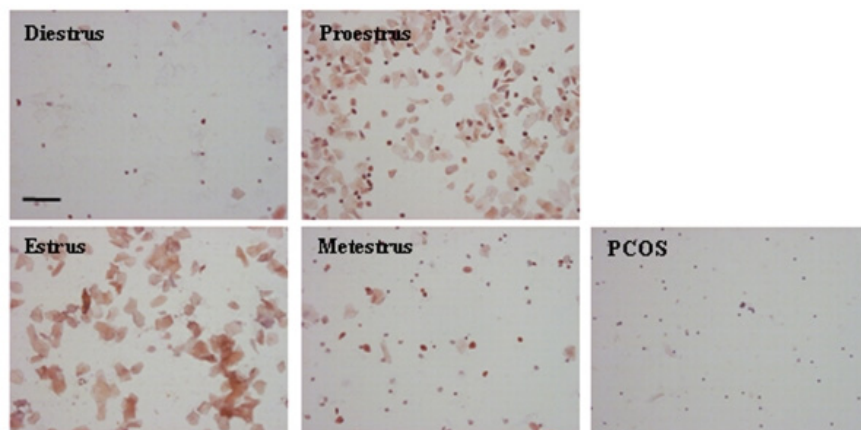

Figure 7. Vaginal smears indicating different stages of estrous cyclicity of the rat including diestrus, proestrus, estrus and metestrus. Pseudo diestrus of DHT-induced PCOS rats. Modified from previously published picture in Feng Y et al. (2009). Hypothalamic neuroendocrine functions in rats with dihydrotestosterone-induced polycystic ovary syndrome: effects of low-frequency electro-acupuncture. PLoS One 4:e6638 (348).

Ovulation frequency (paper II)

Progesterone is produced and secreted from corpus luteum to prepare the endometrium for implantation of an embryo. Circulating levels can therefore be used as a marker of ovulation. In our previous studies self reported menstrual frequency was recorded, but since anovulatory cycles occur, this measure alone cannot be regarded as reliable (349). Measuring progesterone is hence a more trustworthy option

to confirm ovulation. This was made in study II where weekly measurements were performed during the intervention and differences in ovulation frequency between the study groups were evaluated. Since we are missing pre-intervention ovulation data we were unable to elucidate whether ovulation increased although most subjects were anovulatory.

Assessment of body composition

Body composition (paper II)

To evaluate body composition in study II we measured body height, body weight, waist and hip circumference and sagittal diameter and calculated body mass index (BMI)[body weight (kg) / body height^2 (m^2)] and waist-hip-ratio (WHR)[waist circumference/hip circumference]. These measures give a crude estimation of body composition with regard to adiposity and adipose tissue distribution but no information about amount of fat mass or ratio of subcutaneous vs. abdominal visceral fat. Weight and BMI is principally dependent on both fat and muscle content, while it is only the excess fat that leads to co-morbidities such as type 2 diabetes and cardiovascular disease. To identify fat percentage a foot-to-foot bioelectrical impedance system (Tanita, Middlesex, United Kingdom) was used. This technique is based on resistance of electrical currents. Lean tissue has a higher electrolyte and water content than fat mass, hence a better conductor of electrical signals. By inducing a low energy, high frequency signal through an anterior electrode and measuring the voltage drop (resistance) at the posterior electrode, a measurement of total body water, lean mass and fat mass can be obtained. It is highly correlated to waist circumference and BMI and the traditional hand-to foot bioelectrical impedance system (350) but may underestimate body fat percent compared to MRI (351). For more accurate measurements the gold-standard technique magnetic resonance imaging (MRI) is a more distinguished method, which allows area and volume determinations of specific tissue compartments with high precision and reproducibility, with the disadvantage of being much a more expensive and resource-demanding resource.

Dual-energy X-ray absorptiometry (paper III)

In paper III, dual-energy X-ray absorptiometry (DEXA) was used to assess body composition in anaesthetized rats. Obtained information from DEXA includes bone mineral content, body fat and lean tissue mass. These three compartments absorb energy from an X-ray beam based on two different energy levels in a tissue-specific manner. Radiation dose is very low and it is easy to use with low operational costs, making it attractive in clinic. One must keep in mind though that the obtained body fat represents total body fat mass and not total adipose tissue mass (352). It can neither distinguish different fat depots from each other such as MRI can.

Insulin sensitivity tests

Estimating insulin sensitivity may be of importance for many physiological conditions or when evaluating pharmaceutical effects. There are several different methods available and the choice of method can be based on the required accuracy. Methods used in the papers comprising this thesis are the euglycemic-hyperinsulinemic clamp, different insulin sensitivity indexes and the oral glucose tolerance test.

Euglycemic-hyperinsulinemic clamp (paper III)

Whole body insulin sensitivity is commonly measured in clinic for different conditions such as type 2 diabetes, metabolic syndrome, obesity, PCOS and during pregnancy. The golden standard procedure for measuring insulin sensitivity is the euglycemic-hyperinsulinemic clamp technique. It is an invasive procedure measuring the amount of glucose needed to compensate for an increased level of insulin, to avoid hypoglycemia and ensuring glucose levels within a euglycemic range. Insulin is infused at a fixed and predetermined dosage with the aim to raise plasma levels to a higher and stable plateau. Without exogenous infusion of glucose, hypoglycemia would develop rapidly. Addition of a variable glucose infusion with the purpose of keeping glucose levels "clamped" at a predetermined euglycemic level will provide information regarding the amount of glucose that is metabolized per kilo body weight at that fixed insulin level. In summary, the method involves one fixed and predetermined insulin infusion and one variable glucose infusion (353). The clamp technique assumes that basal hepatic glucose production is suppressed when infusing insulin and glucose. In

normal subjects this is true, but in different states of insulin resistance where suppression of glucose production might be impaired, risking that the amount of glucose that is metabolized is underestimated. The degree of insulin resistance is inversely proportional with the peripheral glucose uptake and is termed glucose infusion rate (GIR) or glucose disposal rate (GDR) and is normalized to body weight (mg/kg x min).

In paper III rats underwent the clamp during anesthesia. They were infused with a high bolus dose of human insulin to shut down endogenous insulin and glucose production, followed by a stable lower insulin infusion. Glucose was then administered continuously to achieve a steady state at a euglycemic level. Since most glucose uptake occurs in muscle and not in adipose tissue, and overestimation of insulin resistance may be the case in our obese rats. GIR could in that case have been normalized to lean mass. When computing this, significance between groups remains.

Insulin sensitivity indexes (paper II)

Due to the inherent complexity, the clamp is mainly used in medical research, while other simpler and cheaper techniques and indexes have been developed for clinical use. Alternative methods are glucose tolerance tests or indexes based on fasting glucose and insulin levels such as homeostasis model assessment (HOMA) (354) and quantitative insulin sensitivity check index (QUICKI) (355). These indexes correlate rather well with the clamp in healthy controls, type 2 diabetes and PCOS and are routinely used (356). In paper II HOMA index was calculated from the final blood samples obtained in the morning after the blood sampling for LH pulsatility. The clamp was not performed since assessment of insulin sensitivity was not a primary aim of the paper.

Oral glucose tolerance tests (paper IV)

A glucose tolerance test (GTT) gives a measure of how quickly an administered dose of glucose is taken up and cleared from the blood. It is widely used in clinic for diagnosis of type 2 diabetes and glucose intolerance where fasting- and 2h post-glucose is used for diagnosis. Experimentally, glucose and insulin areas under the curve (AUC) as well as glucose and insulin levels during different time points are also used to assess insulin sensitivity. The glucose can be administered through different routes and doses, but most common is the orally administered glucose tolerance test

(OGTT). In paper IV blood glucose was measured after a 5-h fasting and rats were thereafter given a physiological dose of glucose (2 g/kg) orally by gavage. Blood was collected from the tail after 15, 30, 60 and 120 min and glucose levels were analyzed directly. Plasma samples were collected at 0, 15 and 30 min for insulin analysis by ELISA. Rats were conscious throughout the experiment.

Compared to the clamp, an OGTT provides more information about glucose tolerance than insulin sensitivity, but it is also a more physiological test including glucose uptake and insulin release. In paper IV we did not want an insulin load on the muscle and fat tissues possibly affecting later gene- and protein analyses which would have been the case if we finalized with a clamp. Instead, when using the OGTT it was possible to perform the experiment one week before sacrificing the animals and was therefore more suitable. Different indexes obtained from the OGTT, such as the insulin sensitivity index described by Matsuda (357) used in paper IV, has also been shown to correlate rather well with clamp data (358). For even more information regarding insulin sensitivity, an additional insulin tolerance test to investigate the lowering effect on blood glucose could have been made.

Gene expression

Studying gene expression is a powerful tool in life science research. It can answer questions like; in which tissues or species different genes are active, the level of expression, when they are activated, in validation of transgenic organisms/cells or to measure viral/bacterial load, cancer and resistance development. Techniques includes traditional polymerase chain reaction (PCR) developed in the 1980's, the related real-time PCR and the newer and more refined high-throughput micro-array and RNA-seq techniques. Measuring mRNA levels is one way to indirectly estimate the protein levels within a cell or tissue. It is easier than measuring proteins directly but one must keep in mind that the two can't be completely correlated and it is the protein levels per se that control cellular processes and events. A change in the protein coding transcript must not imply a change in protein levels but it gives an indication and can be hypothesis generating.

Real-time RT-PCR (paper IV)

Real-time reverse transcriptase (RT) PCR is based on the PCR technique, but with mRNA instead of DNA as starting material. As a first step the enzyme reverse transcriptase is used to create complementary DNA is therefore required. Real-time then simply refer to that the DNA amplification is monitored after each amplification cycle instead of after e.g. 40 cycles. This is to enable estimation of the original amount mRNA and not risk that reaction components in concentrated samples will be used up before final analyzes. This is monitored by the addition of a fluorogenic probe that links a change in fluorescence with amplification of DNA and then detects the emitted light. The intensity of the emitted light is related to the amount of amplified DNA (359).

In paper IV we used TaqMan®low density array (LDA) cards (Applied Biosystems, Foster City, USA). These are micro fluidic cards with 8 sample-loading ports each connected to 48 reaction wells containing primers and probes for selected gene assays. The probe in TaqMan assays consists of a sequence complementary to the target sequence with a reporter dye at the 5' end and a quencher at the 3' end. As long as the probe is intact the emission from the reporter dye is quenched. During each amplification cycle DNA polymerase cleaves the probe and releases the reporter dye which then can emit fluorescent light. Each cycle increases the emitted light and is monitored in real-time. A predetermined threshold is used and the cycle where this threshold is reached is called the cycle threshold, C_T. The format allows for analyzes of up to 384 (48x8) different genes but we designed cards with 48 genes with the possibility to load 8 different samples. These genes also included 5 putative reference genes. For quantification of the real-time RT-PCR result two different methods can be used, either the standard curve method with absolute quantification or the $2^{-\Delta\Delta CT}$ method with relative quantification. We used the $2^{-\Delta\Delta CT}$ method for relative comparison between samples (360). In short, the first step was to normalize all genes to a reference gene to compensate for experimental variation, giving the ΔC_T value. An optimal reference gene is stable between tissues and individuals and should not be altered by the experimental setup or design. We used the Normfinder algorithm to find the most stable gene or gene combinations with the lowest intra- and intergroup variability (361). To convert ΔC_T to relative quantities we normalized it to the mean of the untreated group giving $\Delta\Delta C_T$. Finally we transformed it to fold changes $2^{-\Delta\Delta CT}$ with the untreated control group representing mean of 1 and relative numbers for the different treatment groups.

The first steps including mRNA isolation and cDNA preparation are technically challenging and measures must be taken to assure DNA-free, good quality and non-degraded RNA, suitable primers and probes and so on, but correctly used it is considered to be the most sensitive method for quantification of mRNA levels (362). Due to the format of the cards, the same mix of enzymes and cDNA is used for all measured genes, which diminishes pipetting errors, making the method easy to use and very accurate. For further investigation and quantification of your target protein, Western blot may be recommended.

Protein expression

Measuring the amount of protein within a tissue, organ or cell type is widely used and is more descriptive and informative than only looking at gene expression. Looking at the protein level gives you a snapshot of the actual processes that is taking place within the cell at that precise moment. The actual activity of proteins may be dependent to post-translational modifications such as phosphorylations, something gene expression does not consider, but possible to analyze in protein expression methods. However, measuring protein expression are at present time much more time consuming and cumbersome than gene expression. For larger screenings of several targets, gene expression may be used but further confirmation with protein expression is warranted. Protein expression methods are based on the use of antibodies, either polyclonal or monoclonal. Polyclonal antibodies recognizes several different epitopes on a target molecule but can be less specific and cause problems with background staining or false positives. Monoclonal antibodies are instead specifically directed to only one epitope on the target molecule, but are sometimes too specific. They are also more technically demanding and expensive to produce.

Immunohistochemistry (paper I, III-IV)

Immunohistochemistry (IHC) is used to demonstrate the presence or the localization of a target antigen within a tissue. The antigen is recognized by directed antibodies that are both directly or indirectly coupled to different detection molecules, and thereafter visualized in a microscope. It is now a standard assay in pathology although lack of standardization and reproducibility. Quantification of IHC data has been a challenge and lacked a broad acceptance although new systems and software has been

developed (363). One fair interpretation may be that it is semi-quantitative. Different detection methods can be used and most commonly used are colorimetric, and direct or indirect immunofluorescence. Colorimetric/chromogenic methods such as 3,3'-Diaminobenzidine (DAB) staining is commonly used where an enzyme convert DAB into brown colour products. It is sensitive, with a long shelf-life for stained sections but can cause background staining and signal amplification. Immunofluorescence using antibodies coupled to fluorochromes and visualizing in confocal microscope is considered to be more sensitive and specific, with easier interpretation and ability for simultaneous visualization of different targets in e.g. co-localization experiments. Background emission and short time-frames are some of the drawbacks of fluorescence. In paper I DAB staining was used for quantification (cell count of immunoreactive cells) while dual-immunofluorescence staining was used for co-localization experiments. In paper III and IV we used immunofluorescence staining for semi-quantification and protein localization.

Western Blot (paper I, III-IV)

Western Blot, first described by Towbin et al. in 1979, is a widely accepted technique to analyze protein content within tissues or cells (364). The method is based on protein separation by size by gel electrophoresis and thereafter protein transfer by electroblotting to a nitrocellulose or polyvinylidene difluoride (PVDF) membrane. Antibody detection is lastly used for detection of the target protein. Detection methods include colorimetric, chemiluminescent, fluorescent and radioactive probes or substrates. One advantage of PVDF membranes are their inherent resistance to damage and their ability to easier be stripped and re-probed with new antibodies without causing background noise. The main benefit of using western blot is the control of antibody specificity. This is possible since the size of the detected protein band can be compared with the expected protein size and you can also evaluate whether the antibody binds to more than one protein, and if you have degradation products. This information is not included in immunohistochemistry, and by this you can presume that you analyze the correct protein. Western blot is also considered to be more quantitative compared to IHC. For further quantification other methods like ELISA, radioimmunoassay (RIA) or enzyme immunoassay (EIA) may be used. However, in these methods the size of the immunoreactive protein cannot be controlled and the concentration could be underestimated since the antibodies might only recognize correctly folded protein.

Compared to working with DNA and RNA, working with proteins involves challenges due to their very different inherent properties. The specificity and sensitivity of a western blot is also mainly due to the chosen antibodies. Conditioning optimization for each protein *and* antibody is therefore required, and due to the amount of different steps in western blot this can be very time consuming. In paper I, III and IV western blot with chemiluminescent detection was used for protein quantification. We encountered problems like unspecific antibodies, no visible protein bands and high background staining. These were solved by testing different antibodies (both primary and secondary), blocking solutions (skim milk or bovine serum albumin, BSA), concentrations and incubation or washing times.

Hormone pulsatility analyses (paper II)

Several of our hormones and gonadal sex steroids are secreted pulsatile where several regulatory inputs control and generate these pulses. A pulse is defined as a punctuated time delimited event with a marked chronological increase and decrease in the secretion rate. Both the frequency and amplitude can determine the mean concentrations and can be altered in physiological conditions (365). There are now several software's available to calculate the characteristics of pulsatile secretion including Pulse XP and other Matlab algorithms.

In paper II identification and characterization of LH and cortisol pulsatility and secretion pattern were made with Pulse XP software using Autodecon and ApEn applications. AutoDecon is a validated, non-subjective and standardized algorithm for the detection of hormone secretion pulses. Compared to previous versions it is completely automated and does not require estimated input such as number of peaks, positions and amplitudes (366, 367). AutoDecon uses a deconvolution algorithm which means that it separates a hormone concentration time series into the two events: the rate of hormone entry and the rate of hormone removal from the bloodstream. A *fitting* module within the program fits a mathematical model to your experimental data with a weighted nonlinear least-squares algorithm so the parameters of the model have the highest probability of being correct. An *insertion* module then adds a presumed peak at the most probable location. The *triage* module then performs a statistical test whether the presumed peak should be removed or not. This is iterated until no more secretion events are added or removed (367). Some of the time series

contained missing samples due to e.g. clotting of venflon cannula. For more than two consecutive missing samples files needed to be truncated before analysis, hence creating time series of less than 12 h. Since some of the variables were characterized as per 12h they needed to be re-calculated for correct comparison before statistical tests.

Approximate entropy (ApEn) is a statistical method for the evaluation of dominant and subordinate patterns and temporal irregularity of serial data. These patterns do not have to be peak occurrences or amplitudes. It measures the logarithmic likelihood that m contiguous observations that are similar (within distance r) remain close (within r) the next incremental comparison. Monte Carlo simulations of shuffled series are then analyzed to represent a maximal entropy or randomness to which your data could be compared. Larger ApEn mean represent greater randomness or less orderliness while smaller numbers represent more recognizable patterns (368). In paper II we used a window length of m=1 and a tolerance parameter of r = 20% (ApEn: 1, 20%). To calculate standard deviation of approximate entropy in each series 1000 Monte Carlo simulations were used.

Mass spectrometry (paper II)

In paper II levels of sex steroids, androgen precursors and glucuronidated androgen metabolites were analyzed with gas chromatography-tandem mass spectrometry (GC-MS/MS) and ultra performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS). The first part, gas or liquid chromatography, carries the injected mixture in a mobile phase through a heated column containing a solid phase and separates it into pure chemicals. The second part, mass spectrometry, identifies and quantifies these chemicals. As the names suggests in GC the mobile phase is a carrier gas and in UPLC it's a liquid. The benefits compared to immunoassays of using a mass spectrometer as detector are the acquiring of both qualitative and quantitative data. Although the machinery has a high initial cost the specificity and sensitivity as well as the utility with a mass spectrometer is inferior to immunoassays. To measure for example low testosterone levels in females an immunoassay is simply not sensitive enough (369). The use of tandem mass spectrometers basically improves selectivity and sensitivity of the analysis.

Statistics

All statistical analyses were done using SPSS statistical software (SPS inc., Chicago, USA). In paper I, III and IV data is expressed as mean ± standard error of mean (SEM) and in paper II as mean ± standard deviation (SD). $P < 0.05$ was considered significant.

Weight development in paper III and IV was measured by mixed between-within analysis of variance (ANOVA) since weight was considered to be normally distributed. In paper III we wanted to investigate when weight started to differ between a healthy control group and DHT-induced PCOS rats. Therefore, t-tests comparing each time point were also conducted. Other comparisons between individual groups in paper III and IV were done by Mann-Whitney U-tests.

In paper I multiple comparisons between groups were done with one-way ANOVA followed by correction of P-values by Dunnett's post-hoc tests.

In paper II data was analyzed according to the intention-to-treat (ITT) principle. Missing end of treatment data due to dropouts after baseline assessments were replaced using the baseline observation carried forward method. Dropouts occurring before baseline values were collected were excluded from the ITT analyses. All comparisons between the intervention groups were made by Mann-Whitney U-tests since most of the variables were not normally distributed. Differences in the categorical variables acne and menstrual cycle pattern were assessed by the chi-square (or χ^2) test.

KEY RESULTS AND DISCUSSION

All results can be read in their complete in each paper included in the thesis. Here I take the opportunity to highlight and discuss the key results. To retrospect to the aims of this thesis, the results will first be presented and discussed in two parts concerning the mechanistic effects of acupuncture on 1) ovulatory and neuroendocrine dysfunction and 2) metabolic dysfunction.

Effect on ovulatory and neuroendocrine function (paper I-II)

Two of the key characteristics of PCOS are their hyperandrogenemia and ovulatory dysfunction, where the two are mutually interfering with each other to enhance the pathology (222). We have previously demonstrated that electro-acupuncture improves ovarian morphology in the DHT-induced rat PCOS model and improves menstrual pattern in women with PCOS (233, 370). Here we aimed to investigate whether this also applied for ovulation and if this effect worked through mechanisms related to the HPO axis.

Acupuncture improves reproductive ovarian function

Previous experiments have demonstrated improved ovarian morphology after electro-acupuncture in DHT-induced PCOS rats (340). Here, in the same PCOS model, we followed estrous cyclicity during acupuncture treatment by daily vaginal smears. In the rats who received electro-acupuncture we found a re-emerging cyclicity from the constant pseudo-diestrus they otherwise display (39). This was later confirmed in a succeeding study where both electrical and manual stimulation improved cyclicity in DHT-induced PCOS rats, with no significant difference between the two stimulation modalities. Both electrical and manual stimulation of the acupuncture needles also increased progesterone levels, consistent with the improved cyclicity (371). In the clinical situation we determined ovulation frequency in PCOS women, during a 3 month long acupuncture or attention control treatment period, by weekly

progesterone measurement and menstrual bleeding registrations. The group who were allocated to acupuncture treatment had higher ovulation frequency compared with the attention control group, an effect that was augmented compared to previous clinical studies on PCOS with fewer acupuncture treatments (233, 370, 372). In addition, the attention control group had also high ovulation frequency, comparable with ovulation frequency during clomiphene citrate stimulation, as well as a previous acupuncture study in PCOS women (256, 372). The parallel effect on ovulation in the rat and human studies strengthens the results by their translational nature. No major changes was observed in ovarian morphology in the clinical study when counting antral follicles ≤ 9mm or ovarian volume, however a tendency to reduced number of antral follicle and ovarian volume was observed in the acupuncture group. This is in line with our previous animal studies where electro-acupuncture improved ovarian morphology in DHT-induced PCOS rats (340). In addition, in the clinical trial we found reduced circulating levels of inhibin B in the acupuncture group, which may represent decreased size of the follicular pool or explain the prevented follicular arrest and reduced levels of ovarian sex steroids (126-128, 131).

Moreover, delta changes of most circulating sex steroids (E1, E2, E1-S, T, free-T, DHT, sex steroid precursors (DHEA, DHEA-S) and glucuronidated androgen metabolites (ADT-G, AD3G and AD17G), as determined by mass spectrometry differed between the intervention groups. Differences in E1-S, E2, DHEA, free-T and ADT-G held for bonferroni correction. Acupuncture reduced levels of E_1, E_1-S, E_2, DHEA, DHEA-S, 4-DIONE, T, and free T from baseline to end of treatment. This is in line with, and extends, the previous RCT where some of these steroids were reduced (233). Our succeeding experimental data on the DHT-induced rat PCOS model have later confirmed reduced levels of testosterone after electrical stimulation of the acupuncture needles (371). As in previous studies we found no differences in SHGB levels, which could be explained by that we concurrently saw no treatment effect on insulin levels (51, 233). The enhanced effect regarding both ovulation and sex steroids may be explained by increased number of treatments, as our animal studies infer dose-responsive effects (317, 373).

Acupuncture partly improves neuroendocrine function

To further elucidate the underlying mechanisms of electro-acupuncture on the effect on estrous cyclicity in the DHT-induced rat PCOS model the levels and distribution of AR and GnRH were determined. In women with PCOS we aimed to elucidate

whether the effect of acupuncture is mainly of peripheral, ovarian origin or via central control mechanisms. To elucidate possible explanations for the effect of acupuncture on ovulation in PCOS women we measured LH pulsatility and secretion by a 12 h blood sampling procedure.

Altered hypothalamic protein expression in DHT-induced PCOS rats

In the rat, the highest density of GnRH neurons is located in the rostral medium septum (MS), diagonal band of Broca and medial preoptic area (MPO) (56). Most GnRH neurons send projections via mediobasal hypothalamus down to the median eminence where GnRH is released in to hypophyseal portal blood (374, 375). In our study, we found more GnRH-immunoreactive (GnRH-ir) cells in the MPO and horizontal limb of the diagonal band (HDB) in the PCOS rats. Additionally, compared to controls the PCOS rats displayed increased levels of hypothalamic functionally active AR and AR-ir cells in the MPO as determined by western blot and IHC. This implies that androgens have a regulatory effect on the GnRH neurons. Moreover, the increased levels of GnRH-ir cells and AR in hypothalamus were reduced after electro-acupuncture treatment. Estrogen receptor β has previously been shown to be co-localized with GnRH neurons, thereby indicating a direct action of estrogens on GnRH regulation (376), but no one had reported presence of AR in GnRH neurons. Here we demonstrated a co-localization of AR and GnRH neurons that further strengthens the hypothesis of a direct androgenic regulatory control on GnRH neurons. Hence, we have indications that both the GnRH abnormalities as well as the effect of electro-acupuncture could be mediated via the AR in hypothalamus. Moreover, previous experiments have demonstrated that levels of circulating estradiol is not altered in the DHT-induced rat PCOS model, which further supports the conclusion that we are looking at an androgenic effect (39).

Although arcuate nucleus is considered to the principal location of the GnRH pulse generator in the rat brain, the preoptic area regulates the GnRH secretion surge controlling the preovulatory LH surge (299, 377) making our findings in MPO even more interesting. Administration of androgens prenatally have previously been shown to increase LH pulsatility, without affecting pituitary responsiveness to GnRH, and to abolish the estrogen induced LH surge, which is indicative of acceleration in the GnRH pulse generator. A contemporary reduction of estradiol induced progesterone receptor (PR) expression in the preoptic area was then considered evidence of how androgens could mediate this effect (330). 4-day of androgen administration to adult rats produced similar results on the LH surge and PR expression, but with the

contrast of an instead decreased LH and possibly GnRH secretion, similar to the male response (378, 379) and in oppose to human PCOS. These results are indicative of a programming effect (defect) of androgens during prenatal development that is different from the direct effects of hyperandrogenemia in adult life. Since our rats are exposed to androgens on day 21, it is possible that they have reduced GnRH secretion, with abolished GnRH/LH surges (378, 379). One might therefore speculate that restoration of the GnRH and LH surge by effects on the androgen receptor in the MPO could be, at least part of, the mechanism behind the improved estrous cyclicity after electro-acupuncture. Our succeeding study could not confirm the affected hypothalamic GnRH and AR expression, but there we only measured mRNA and not protein expression (371). This could mean that the effect is related to post translational events rather than gene expression changes.

Lack of effect on LH-pulsatility in PCOS women

The effect of acupuncture on the hypothalamic aberrations in the DHT-induced rat PCOS model and the initial finding that 14 acupuncture treatments improve menstrual bleeding pattern and reduced circulating total testosterone made us wonder whether the improved cyclicity in PCOS women is due to restoration of gonadotropin secretion or whether it is an effect at the ovarian level. To attend this hypothesis in the clinical situation we analyzed LH pulsatility in our PCOS women. Since LH pulsatility is known to decrease by obesity (72), we only included women with a BMI less than 30.

None of the LH pulsatility measures were affected by acupuncture treatment twice a week during 3 months. Instead the major effect accompanying the higher ovulation frequency and reduced circulating inhibin B levels in the acupuncture group was the general decrease in circulating sex steroids, androgen precursors and glucuronidated androgen metabolites after treatment, again pointing towards an effect at ovarian and adrenal level. However, it does not exclude involvement of central control mechanisms.

The lack of effect on LH pulsatility and secretion pattern may also be due to the statistical limitation caused by too many drop outs. It could otherwise simply mean that the mechanism is concealed elsewhere. One reason behind the lack of effect on the LH pulsatility and secretion pattern may also be the sampling timing in the menstrual cycle, which could be a confounding factor (81, 262, 380). In a normal menstrual cycle, the LH frequency and amplitude increases in late follicular phase

(381, 382) and is more similar to the constant high LH frequency and amplitude in found PCOS. The majority of endpoint overnight blood sampling was performed during cycle day 8–10 (mid to late follicular phase), and only 6 were performed independent of cycle day (4 in the control and 2 in the acupuncture group). It is possible that the if the higher ovulation frequency induced by acupuncture was due to a normalization of gonadotropin secretion with a conventional slowing of the LH pulsatility frequency during the luteal and early follicular phase followed by a normal increase in pulsatility in the late follicular phase, a possible treatment effect might have been more prominent if blood samplings were performed in early follicular phase. In a recent RCT comparing sham and true acupuncture in PCOS women the LH:FSH ratio was decreased in both groups together with rather high ovulation frequencies (372). Our data reflected a similar difference for both LH and LH:FSH ratio when comparing delta change between groups. But our LH pulsatility and secretion measures, despite randomization, incorporated baseline group differences in these two measures. When we corrected for these differences we found no treatment effect.

Conjunction point for reproductive and neuroendocrine effects

The causality of the presented data regarding changes in ovulation and sex steroids is difficult to determine. The reduction of androgen levels by normalization of intrinsic ovarian aberrations may calm the exaggerated follicular growth, restore the follicle maturation processes and hence lead to ovulation (50). It could also be the other way around, that improved ovulation, possibly caused by external factors, restores follicular growth and/or aberrations and thereby reduces androgen levels due to the lower pool and/or activity of androgen producing cells (68). This is in line with the tendency towards reduced number of antral follicles and ovarian volume after acupuncture. We performed correlation analyses between ovulation and endpoint variables, in the whole material, to see which variables that were associated with ovulation. Estrone, estradiol and androstane-3α, and 17β-diol-3-glucuronide were all associated with ovulation, but none of the LH variables. The involvement of sympathetic nervous system and an alteration in neurogenic control of the ovary has been implicated in PCOS etiology (278, 279, 383, 384). One hypothetical mediator for that effect, combining an autonomic and ovarian effect, is nerve growth factor (NGF). Ovarian NGF production is increased in the follicular fluid in women with PCOS and in an estradiol valerate induced rat PCOS model (213, 385, 386). Overexpressing NGF in the ovaries of a transgenic mice model results in ovarian hyperinnervation, arrested follicle growth and increased ovarian steroidal responsiveness to

gonadotropins (213). Electro-acupuncture has been shown to decrease high ovarian NGF in the estradiol valerate PCOS model (385, 386), as well as decrease several markers of sympathetic activity in adipose tissue of the DHT model (340). Together with our microneurography measurements demonstrating decreased high muscle sympathetic activity after 14 acupuncture treatments, this indicates the involvement of the sympathetic nervous system in both PCOS etiology and the mechanistic effect of acupuncture (217). β-endorphin, an opioid, is known to modulate sympathetic tone (387, 388) and have inhibitory effect on GnRH release, and GnRH secretion has been shown to be increased in PCOS (288, 291, 298). That naltrexone, a μ-receptor antagonist induces ovulation and decreases LH concentration further indicates the role of β-endorphin in PCOS (310-312). That similar effects are also mediated by electro-acupuncture (314-316) concurrent with a lowering of β-endorphin levels (314) implies the involvement of the opioid system in the underlying mechanism. This is also supported in our recent experimental study where electrical stimulation of the acupuncture needles affected the expression of opioid receptors μ and ϰ in rat hypothalamus, together with improved cyclicity and reduction of testosterone levels (371). That acupuncture had an effect on both adrenal and ovarian originating sex steroids in paper II favor a central mechanism controlling the effect of acupuncture.

Altogether, both the clinical and experimental data in this thesis support that acupuncture has a beneficial effect on ovulation which is related to decreased levels of sex steroids, their precursors and glucuronidated androgen metabolites, and inhibin B. Although the clinical data does not support changes in LH pulsatility/secretion pattern as a possible mediator for this effect, there is strong evidence of central components that probably involve both opioid and sympathetic activity, an effect that most likely is mediated via the androgen receptor.

Acupuncture on metabolic function (paper III-IV)

The association between PCOS and insulin resistance and compensatory hyperinsulinemia, and the increased risk of type 2 diabetes and possibly CVD definitely demands attention (42, 167-169, 172). Insulin resistance, occurring also in the lean PCOS women and aggravating both endocrine and metabolic features and the effect of acupuncture were here investigated in the DHT-induced rat PCOS model. We have previously demonstrated improved insulin sensitivity after both acupuncture and exercise (317). Here we aimed to investigate the effect of more

frequent acupuncture treatments on insulin sensitivity and to compare the effect of manual and electrical stimulation of the acupuncture needles.

Acupuncture improves insulin sensitivity

Whole body insulin sensitivity and glucose tolerance in the DHT-induced rat PCOS model was measured with the golden standard euglycemic hyperinsulinemic clamp method and the oral glucose tolerance test (OGTT). Rats responded to electro-acupuncture treatment five days per week with a normalization of insulin sensitivity, measured by the clamp, with no concurrent improvements in body composition. This was an enhancement of the effect seen in the previous experiments when animals were treated three days per week (317). Next we aimed to elucidate whether low-frequency electrical acupuncture stimulation used in the previous trial is more effective than manual stimulation of the needles in the improvement of insulin sensitivity. In this experiment we avoided the clamp since we aimed to analyze protein expression in a basal non-insulin stimulated state. Instead we used the OGTT where we surprisingly enough found no treatment effect after electrical stimulation. Previous studies have reported improved glucose tolerance, although acutely and in male rats (389-391). However, after electrical stimulation we saw a decrease in inguinal fat mass. After manual stimulation we found no effect on OGTT AUC but decreased fasting and 120-min glucose levels as well as increased glucose clearance, without changes in insulin levels. This implies at least partly improved glucose tolerance and /or improved hepatic insulin sensitivity or decreased pancreatic glucagon release by manual acupuncture. It might also be reflective of improved insulin sensitivity. The discrepancy between the clamp and OGTT data in the same DHT-induced rat PCOS model may have several causes. Primarily, although OGTT data is correlated with clamp measured insulin sensitivity, it doesn't have the precision for quantitatively measure insulin sensitivity, and includes both glucose effectiveness and insulin sensitivity in oppose to the clamp which only measures the latter (192, 357, 392). Other explanations may be the different levels of insulin as well as the duration of insulin stimulation in the two experiments. The high insulin levels in the clamp may reflect improved insulin responsiveness, while the probably lower insulin levels in the OGTT reflect no change in insulin sensitivity or glucose tolerance by electrical stimulation of the acupuncture needles. The long duration of the clamp is also more likely to suppress hepatic glucose production and avoid underestimation of the amount of glucose that is metabolized. Another issue is that in the OGTT we did not take cycle stage into account as we did in the clamp experiment. Since insulin

sensitivity is known to fluctuate over the cycle this may be a confounding factor (346, 347).

Acupuncture affects molecular signaling pathways

Insulin or contraction stimulated glucose uptake in skeletal and adipose tissue is dependent on GLUT4 translocation to the plasma membrane (183, 393). In paper III we measured gene and protein expression of some key molecules in the insulin signaling cascade. After electro-acupuncture we found increased insulin stimulated GLUT4 protein expression, both in the plasma membrane and cytosol of skeletal muscle cells, as measured with western blot and immunohistochemistry. Moreover, no change in Akt or AS160 activation was recognized. This indicated that the improved insulin sensitivity was regulated by increased GLUT4 translocation capacity, but perhaps not via the traditional insulin signaling pathway. Levels of GLUT4 protein after acute electro-acupuncture have previously been shown to be increased in insulin resistant male rats, without insulin stimulation (394). On the other hand, a possible change in AKT and/or AS160 activation might have been masked by the maximum stimulation of high insulin levels in the clamp (199, 200).

In the following study (paper IV) in which we compared electrical and manual acupuncture stimulation, we measured gene and protein expression in both skeletal muscle and adipose tissue, in a non insulin-stimulated state. The final acupuncture treatment was given one hour before tissue collection. A summary of the western blot results of paper IV is presented in figure 8. Although we found no effect of on glucose tolerance by electrical stimulation, protein expression of several key molecules related to insulin sensitivity was regulated in the non insulin stimulated state. Levels of GLUT4 protein was measured with gene expression, western blot and IHC analyses. No changes were detected on GLUT mRNA expression levels, neither after manual nor electrical stimulation in adipose tissue or skeletal muscle. Moreover, total GLUT4 protein content, measured by western blot, revealed no changes in adipose tissue after either treatment modalities. GLUT4 protein content in skeletal muscle, on the other hand, was decreased by electrical but not manual stimulation, indicating lower glucose transport after electrical stimulation. However, GLUT4 protein content measured by IHC revealed increased expression in all cell compartments in skeletal muscle after both manual and electrical stimulation, with more intense signal after electrical stimulation. No such effect was seen in adipose tissue. Therefore, we hypothesized that electrical stimulation has stronger effect on translocation than expression in

skeletal muscle, with no effect on adipose tissue. The discrepancy to the increased GLUT4 protein expression in skeletal muscle after the clamp may be explained by the clamps high insulin levels of long duration, driving also protein expression.
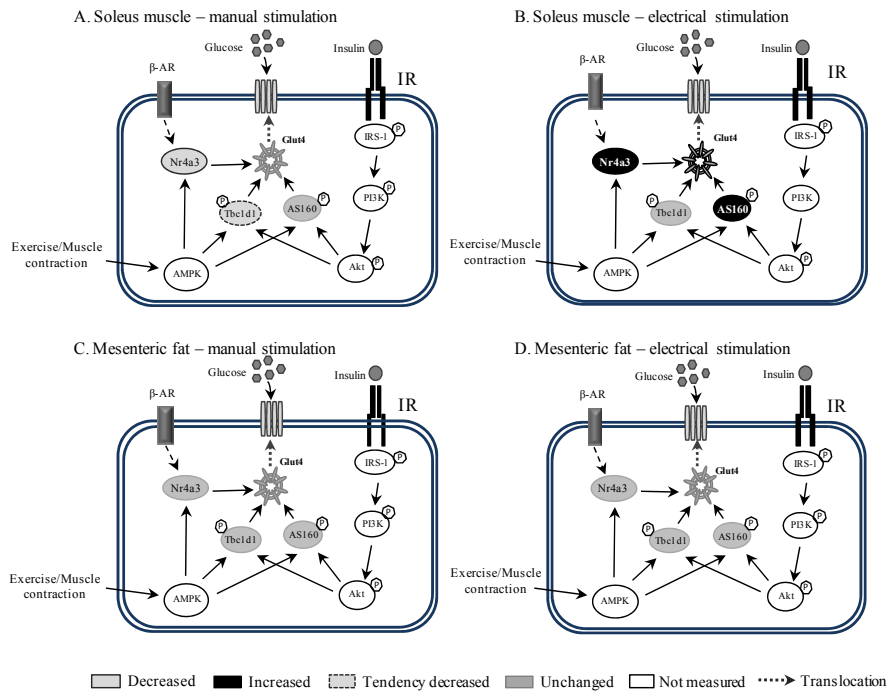

Figure 8. Summary of non-insulin stimulated western blot results on Nr4a3, Tbc1D1 and AS160 after 4-5 weeks of manual and electrical acupuncture in dihydrotestosterone treated female rats. Akt: protein kinase B , IRS-1: insulin receptor substrate 1, GLUT4: Glucose transporter 4, PI3K: phosphatidylinositol 3-kinase, AMPK: 5' adenosine monophosphate-activated protein kinase.

Protein levels of Nr4a3 and activation of AS160 (pAS160/S160 ratio), proteins involved in GLUT4 translocation, was simultaneously increased in skeletal muscle by electrical acupuncture stimulation. Nr4a3 has been implicated in insulin sensitivity and oxidative metabolism, and increased levels aid insulin to stimulate glucose transport and GLUT-4 translocation. Contractile activity has also previously been shown to increase mRNA levels of Nr4a3, probably mediated by AMPK, which supports our results (395-397). Similar results are reported after treatment with β-adrenergic agonists indicating a sympathetic control (397). As mentioned before, AS160 may be the link where insulin signaling and insulin independent signaling for glucose uptake converge (177, 184). Our findings of increased levels of both Nr4a3 and AS160 in

skeletal muscle after electrical stimulation, in the basal non insulin-stimulated state, is therefore in compliance with improvements in several theoretical pathways of insulin-independent glucose uptake. After manual stimulation we found decreased protein levels of Nr4a3 in mesenteric fat, but this was not supported by gene expression and its functionality may be questioned since we found no change in GLUT4 expression or translocation.

Efficacy and mechanisms of manual and electrical stimulation

The mechanism of acupuncture on the metabolic function is highly speculative, but it has been related to adrenal β-endorphin secretion to stimulate insulin secretion, cholinergic nerves, pancreatic nitric oxide synthase and somatic afferent nerves (391, 398, 399). Our results indicate that the effect of acupuncture on insulin sensitivity seems to be directed to skeletal muscle. However, we did see increased gene expression of MAPK3, Glycogen synthase kinase 3 beta (Gsk3b) and adenylate cyclase 3, proteins all involved in insulin sensitivity (400-403), in adipose tissue although these were not confirmed by western blot measurements. Therefore, we cannot exclude that the improved insulin sensitivity also involves regulatory mechanisms in adipose tissue. When evaluating the magnitude of manual and electrical stimulation and the effect on insulin sensitivity in the current setting we found stronger beneficial effects of electrical stimulation, with support from the clamp, protein and IHC data. On the other hand, manual stimulation had a partly positive effect on glucose tolerance and on gene expression in adipose tissue although we found no functional molecular evidence to support the mechanism. Adding up with our later data on estrous cyclicity where we did not see a difference between the groups after the two stimulation modalities we cannot consider electrical stimulation to be superior than manual in all aspects (371). Importantly, the differences between the two stimulation modalities could reflect differences in stimulation duration, given that needles were manually stimulated every 5 min by compared with continuous low-frequency electrical stimulation. Of importance to mention is that the results presented in this thesis regarding insulin sensitivity is only based on animal experimental data and not on clinical evidence, in contrast to the results on reproductive function. Our previous clinical study measuring insulin sensitivity with the euglycemic hyperinsulinemic clamp found no effect of either acupuncture or exercise (404). This may be due the low intensity and frequency of the interventions. It remains to elucidate whether an increased number or treatment frequency, could affect metabolic variables such as insulin sensitivity.

GENERAL DISCUSSION

The data presented in this thesis show that acupuncture have beneficial effects on both reproductive and metabolic function, without any effect on anthropometrics as an explanatory factor. This is derived from both experimental animal data and a human PCOS study. Importantly, the main difference between these two experimental studies is that the DHT-induced PCOS rats are mainly hyperandrogenic, since DHT cannot be converted to estrogen, and therefore we focus on the alterations that are represented by androgens. Human PCOS is more complex with possible effects from estrogens and androgen metabolites. Therefore, the conclusions from the animal data cannot be completely translated to the clinical situation. On the other hand, the animal studies gave us the opportunity to investigate the androgenic effect within the hypothalamus. This provided us with evidence that androgens may influence GnRH secretion and that acupuncture regress these alterations which may explain improved estrous cyclicity. The acupuncture situation is also easier to control for when using animals, since they don't have the influence of treatment expectancies. When designing a clinical study it is of importance to decide what to control for. In pharmaceutical studies the standard procedure is to use a placebo. For non-pharmaceutical interventions such as exercise, acupuncture and most likely psychotherapy the control is more complicated where a true placebo is not available. In paper II we decided to control for the time and attention involved in the therapeutic meeting of acupuncture treatments. We used an attention control group instead of any "placebo needle" or minimal acupuncture treatment that is not completely inert, but neither with this solution we implicate that it is placebo controlled. In paper IV we chose to mimic the clinical situation, where acupuncture treatment is performed by a combinatory treatment where some needles are stimulated electrically and some manually. Electrical stimulation of needles is normally continuous while the manual needle stimulation is intermittent and we aimed to elucidate if those two treatments have different mechanistic effects. Our present data and the subsequent study indicate that electrical stimulation may be more effective than manual stimulation, but perhaps not in all aspects (371). We cannot though exclude that these results are mainly time-dependent due to the intermittent nature of manual stimulation, especially since we also see that the effect of acupuncture is dose-responsive (233, 317, 373). To further elucidate the efficacy one would have to

perform intermittent electrical stimulation equalized to the manual stimulation or continuous manual stimulation. Another issue to discuss is the treatment frequency. We gave our DHT-induced PCOS rats acupuncture 5 days per week and the women twice per week. Neither of these treatment frequencies are probably achievable long term in clinic. On the other hand, even though the effect appears to be dose-dependent, when looking at previous data, we also see that it is long-lasting (233, 404). This implies that a period with more frequent treatment to gain a wanted effect may well be followed by less frequent maintenance treatments as usually common in the clinic.

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

Science is nothing but perception, Plato said. I try to keep in mind that our "truth" is simply the best way of describing the data we currently possess. Science is a field of constant update, where hypotheses and theories are established simply to later be rejected. One cannot know how long our truth will stand unchallenged. Furthermore, lack of an effect does not necessary equals no effect.

The results of the papers presented in this thesis indicate that acupuncture with manual and electrical stimulation elicits local and systemic effects on insulin sensitivity and reproductive function and may represent a supplement or alternative to the traditional treatments. The key results of this thesis are:

- Acupuncture improves ovulation frequency and regulates sex steroid production rather than gonadotropin secretion in PCOS women.

- Electro-acupuncture in DHT-induced PCOS rats improves estrous cyclicity. Moreover, there is a co-localization of the nuclear androgen receptor with GnRH in the hypothalamus, and the effect of acupuncture is related to normalization of hypothalamic aberrations that probably are mediated by androgen receptor activation.

- Both electrical and manual stimulation have beneficial effects on metabolic function in DHT-induced PCOS rats. Electro-acupuncture normalizes insulin sensitivity and regulates insulin sensitivity related protein expression, while manual acupuncture partly improves glucose tolerance but do not regulate key signaling pathways to the same extent.

- The present data indicates that electrical stimulation is superior to manual stimulation, although the effects may be mediated via differential regulatory mechanisms.

The viscous circle, driven by androgens, insulin or other factors, must be broken to improve the health status of women with PCOS. Although pharmaceutical alternatives may be effective, they are also associated with negative side-effects (264). The results of this thesis further strengthen the idea of acupuncture as a potential treatment option for both the reproductive and metabolic disturbance in PCOS. Although we here provide some answers there are more questions to be dealt with. We have repeatedly shown in both clinical and animal experimental studies that acupuncture is beneficial for ovulatory dysfunction in PCOS. For the purpose of implementing acupuncture in the conventional treatment strategy, though, it is of importance to compare it with the first line pharmaceutical options. This is important for the support and sanction of acupuncture as a treatment method. We are now involved in a multicenter clinical study comparing the effect of acupuncture versus clomiphene citrate on live birth rate and ovulation in anovulatory women with PCOS that will address this question. The effect on metabolic function has only, but repeatedly, been demonstrated in animal experimental studies. We have now started a clinical study in which we aim to translate our experimental findings into women with PCOS with the hypothesis that acupuncture improve insulin sensitivity and regulates key signaling molecular pathways in skeletal muscle and adipose tissue. To further investigate the underlying mechanisms of the beneficial effects may also be supportive in the search for other possible alternative treatments, including pharmacological. The mechanism is probably dual with one acute effect and another long term. The data in this thesis are representative of the long term mechanism, but we are currently conducting experimental studies where we investigate the acute effects on insulin sensitivity of both manual and electrical acupuncture. As mentioned earlier studies comparing intermittent electrical with manual stimulation could also better answer the efficacy of the two. There is evidence that the effect of acupuncture is mediated via the opioid and sympathetic nervous system (210, 307, 314). To further investigate the involvement of the sympathetic, parasympathetic and opioid system we also plan to perform acupuncture studies where we will block these different systems.

Altogether, the current thesis has demonstrated that acupuncture have beneficial effects on reproductive and possibly also metabolic function and may represent a therapeutic option for PCOS. Continuously, more and more evidence are gathered,

supporting the efficacy of acupuncture. Interesting it will be, to follow/be part of this development, with the possible use of acupuncture as a represented treatment in PCOS.

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