From the Department of Clinical Science,
Division of Obstetrics and Gynecology
and
the Department of Medicine,
Division of Endocrinology and Metabolism
Karolinska Institute, Stockholm, Sweden

# POLYCYSTIC OVARY SYNDROME

**A study of adipocyte lipolysis in relation to endocrine and metabolic status**

by

Ingvar Ek


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# ABSTRACT


**Polycystic ovary syndrome**

***A study of adipocyte lipolysis in relation to endocrine and metabolic status***

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The aim of the present thesis was to obtain insight into possible regulatory effects of androgens on the regulation of lipolysis in subcutaneous as well as visceral adipose tissue in women with the polycystic ovary syndrome (PCOS).

In nonobese women with PCOS, adrenergic lipolysis was investigated in both subcutaneous abdominal and visceral adipose tissue and several, novel disturbances were found. (Paper I,II & V)

In subcutaneous adipose tissue (Paper I & II), there was a markedly impaired lipolysis, due to ~10-fold decreased $\beta_2$-AR sensitivity as well as 50% reduced β2-AR density and a novel mechanism in the PKA-HSL complex (Paper II), together reducing the activation of hormone sensitive lipase (HSL). Combined oral contraceptives (OC) therapy did not change the impaired lipolysis (Paper I). The visceral adipose tissue lipolysis (Paper V) was enhanced by again stoichiometric changes in the PKA complex subunits.

These abnormalities promotes accumulation of fat in abdominal subcutaneous depot and "burn off" fat in the visceral depot, thus exposing the liver to a high FFA flux, which could contribute to dyslipidemia and hepatic insulin resistance. This is supported by anthropometric data on fat cell size and computed tomography (CT) of fat depots.

In obese subjects with PCOS (Paper IV), weight reduction was more effective than oral contraceptives in restoring at least in part, some defects in lipolysis in subcutaneous abdominal tissue. This indicates that in obese subjects, insulin resistance seems more important, than sex steroids in regulation of lipolysis.

However in lean healthy women, ovarian downregulation (Paper III), showed an impairment of catecholamine lipolysis, due to 3-fold decreased $\beta_1$-AR sensitivity, without affecting insulin sensitivity or the lipolysis responsiveness, speaking for a complex role of sex steroids in regulation of adipose tissue lipolysis.

Further investigations are needed to clarify the relationships between the different sex steroids in the regulation of lipolysis, both in PCOS and in healthy women.



**Key words:** adipocyte, β-adrenoceptors, FFA, hyperandrogenism, HSL, insulin resistance, lipolysis, PCOS, PKA

*To my mother and father*

## LIST OF PUBLICATIONS

I. **Ek I, Arner P, Bergqvist A, Carlström K, Wahrenberg, H.** 1997 Impaired Adipocyte Lipolysis in Nonobese Women with the Polycystic Ovary Syndrome: A Possible Link to Insulin Resistance? J Clin Endocrinol Metab. Apr;82(4):1147-53

II. **Faulds G, Rydén M, Ek I, Wahrenberg H, Arner P.** Mechanisms behind Lipolytic Catecholamine Resistance of Subcutaneous Fat Cells in the Polycystic Ovary Syndrome. J Clin Endocrinol Metab 2003 May;88(5):2269-73

III. **Ek I, Arner P, Rydén M, Carlström K, Wahrenberg H.** Effects of Down Regulation with GnRH-a on Adrenergic Lipolysis in Healthy Regularly Menstruating Women. (Submitted to J Clin Endocrinol Metab. May 2003)

IV. **Wahrenberg H, Ek I, Reynisdottir S, Carlström K, Bergqvist A, Arner P.** 1999 Divergent Effects of Weight Reduction and Oral Anticonception Treatment on Adrenergic Lipolysis Regulation In Obese Women with the Polycystic Ovary Syndrome. J Clin Endocrinol Metab. Jun;84(6):2182-7

V. **Ek I, Arner P, Rydén M, Holm C, Thörne A, Hoffstedt J, Wahrenberg H.** 2002 A Unique Defect in the Regulation of Visceral Fat Cell Lipolysis in the Polycystic Ovary Syndrome. Diabetes Feb;51(2):484-492

# CONTENTS

# LIST OF ABBREVIATIONS

| | |
|---|---|
| **17-OHP** | 17α-hydroxy progesterone |
| **A-4** | 4-androstene-3,17-dione |
| **ACTH** | Adrenocorticotropic hormone |
| **ADA** | Adenosine deaminase |
| **AMP** | Adenosine monophosphate |
| **AR** | Adrenoreceptor |
| **ATP** | Adenosine triphosphate |
| **BMI** | Body mass index |
| **CAH** | Congenital adrenal hyperplasia |
| **cAMP** | Cyclic adenosine monophosphate |
| **CT** | Computerized tomography |
| **CYP** | Cytochrome P |
| **CYP** | Cyanopindolol |
| **DHEA** | Dehydroepiandrosterone |
| **DHEAS** | Dehydroepiandrosterone sulfate |
| **DHT** | 5α-dihydrotestosterone |
| **$E_2$** | Estradiol-17β |
| **EC50** | Agonist concentration producing half maximum effect |
| **EGF** | Epidermal growth factor |
| **FF** | Follicular fluid |
| **FFA** | Free fatty acids |
| **FSH** | Follicle-stimulating hormone |
| **fT** | Free testosterone |
| **fT3** | Free triiodothyronine |
| **fT4** | Free thyroxine |
| **GDP** | Guanosine diphosphate |
| **GH** | Growth hormone |
| **GLUT** | Glucose transport protein |
| **GnRH** | Gonadotropin releasing hormone |
| **GnRH-a** | Gonadotropin releasing hormone analog |
| **GSK** | Glycogen synthas kinase |
| **GTP** | Guanosine 5'-triphosfate |
| **hCG** | Human chorionic gonadotropin |
| **HDL** | High density lipoprotein |
| **HIEC** | Hyperinsulinemic clamp |
| **HOMA** | fP-Insulin x fP-glucose x $22.5^{-1}$ |
| **HSL** | Hormone sensitive lipase |
| **IGF** | Insulin-like growth factor |
| **IGFBP** | Insulin-like growth factor binding protein |
| **IRS** | Insulin receptor substrate |
| **ITT** | Insulin tolerance test |
| **KRP** | Krebs Ringer Phosphate |
| **LH** | Luteinizing hormone |
| **LPL** | Lipoprotein lipase |
| **MAPK** | Mitogen-activated protein kinase |
| **MFO** | Multifollicular ovaries |
| **MRI** | Magnetic resistance imaging |
| **NIDDM** | Non insulin dependent diabetes mellitus |
| **OC** | Combined oral contraceptives |
| **OD** | Optical density |
| **PAO** | Polycystic-appearing ovaries |
| **PCO** | Polycystic ovaries |
| **PCOD** | Polycystic ovary disease |
| **PCOS** | Polycystic ovary syndrome |
| **$pD_2$** | Negative logarithm of EC50 |
| **PDE** | Phosphodiesterase |
| **PG** | Prostaglandin |
| **PI** | Phosphatidyl inositol |
| **PKA** | Protein kinase A |
| **PKC** | Protein Kinase C |
| **PPAR** | Proliferator-activated receptor |
| **PRL** | Prolactin |
| **PVDF** | Polyvinylidine fluoride |
| **RIA** | Radioimmunoassay |
| **SHBG** | Sex hormone-binding globulin |
| **T** | Testosterone |
| **TG** | Triglycerides |
| **TNF** | Tumor necrosis factor |
| **TSH** | Thyroid stimulating hormone |
| **VLCD** | Very low calorie diet |
| **VLDL** | Very low density lipoprotein |
| **VNTR** | Variable number of tandem repeats |
| **WHR** | Waist/hip ratio |

# 1 POLYCYSTIC OVARY SYNDROME (PCOS)

## 1.1 INTRODUCTION

Polycystic ovary syndrome (PCOS) is a common endocrine disorder of premenopausal women characterized by chronic anovulation, infertility, biochemical and / or clinical evidence of hyperandrogenism and enlarged polycystic ovaries (1-3). Its etiology remains unknown. Few studies have attempted to define its prevalence and an estimated prevalence of 5-10% in women of reproductive age is probably a reasonable conservative figure (2;4-8). PCOS is not only the most frequent cause of anovulation and hirsutism, but is also associated with a characteristic metabolic disturbance (resistance to the action of insulin) (2;9-11) leading to known biochemical disturbances in the metabolism of carbohydrates as well as lipids and sex steroid (11). This may have implications for long term health (12-16). There is also a striking resemblance to the so called "insulin resistant syndrome" or "the metabolic syndrome" affecting both men and women (17-21) with, among other metabolic derangements, glucose intolerance, hypertension, hypertriglyceridaemia and low serum HDL-cholesterol levels and upper body obesity – a global problem in the western world of today.

Many studies have been focused on the cause of insulin resistance in PCOS (11) but there are few investigators studying the adrenergic lipolysis in PCOS despite the close resemblance to the "metabolic syndrome" and its close relation to the adipose tissue. The general aim of this thesis is to investigate the adrenergic lipolysis and its regulation in PCOS.

## 1.2 DIAGNOSIS AND CLINICAL FEATURES

When first described by Stein and Leventhal (22) in 1935 the syndrome was defined by ovarian enlargement and multiple small cysts, in association with amenorrhea, obesity and hirsutism. Subsequently after successful wedge resection (23) of the ovaries in women diagnosed with Stein-Leventhal syndrome, menstrual cycles became regular and these patients were able to conceive. Consequently, a primary ovarian defect was thought to be the main problem, and the disorder came to be known as polycystic ovarian disease (PCOD)(24). Later, as the diagnostic methods of hormonal analysis developed, the interest for hormonal changes in PCOS became greater than the ovarian morphology and the morphology of the ovaries was not considered necessary for the diagnosis(2;25). High-resolution ultrasonography which image ovarian morphology is now needed for basal diagnosis and investigations in PCOS (26-33). In recent years, varying definitions of this syndrome have been used in studies of this disorder, with some investigators requiring polycystic ovaries on (transvaginal) ultrasound for inclusion and other requiring an elevation of serum LH or LH:FSH ratio (34). Further biological, clinical, and endocrinological studies have shown an array of underlying abnormalities; hence, the condition is now referred to as polycystic ovary syndrome (PCOS), although it may occur in women without "ovarian cysts".

A uniform definition of PCOS does not exist, in large part because of its diverse and heterogeneous nature. The most widely accepted clinical definition of the polycystic ovary syndrome is the association of hyperandrogenism with chronic anovulation in women without specific underlying diseases of adrenal or pituitary glands (2). However, milder forms of PCOS that includes women who have hyperandrogenism and polycystic ovaries but whose ovulatory function is maintained have been proposed (35). These women share many of the risks as women with more classic PCOS. While PCOS occurs in at least 5% of the population, the isolated finding of polycystic-appearing ovaries (PAO), which meets the classic PCOS ultrasonographic criteria (27)(Fig.1), of at least 8 follicles of less than 10 mm diameter in one plane, usually peripherally arranged, sometimes with increased amount of stroma, occurs in approximately 20% of the normal population (8). Even these women have enhanced sensitivity to exogenous gonadotrophins during ovulation induction, which is typical of women with classic PCOS, and their ovarian cells produce more androgen than normal *in vitro* (36;37). PAO or PCO (referring only to the ovarian morphology) is known to occur as well in hypothalamic amenorrhea and in CAH, where its prevalence is virtually 100% (38). Anovulation causes sub/infertility and is also seen as amenorrhea, oligomenorrhea, or dysfunctional uterine bleeding. Hyperandrogenism is characterized clinically by hirsutism, which are a function of target tissue sensitivity to androgens and may be absent despite substantial hyperandrogenemia (39). Hyperandrogenism also causes acne, and androgen-dependent alopecia. Biochemically sees elevated serum concentrations of androgens, particularly testosterone (T) and androstenedione (A-4) and often increased serum levels of LH, although normal serum concentrations of LH do not rule out the diagnosis, and low tonic levels of FSH. Obesity is common but not usually a presenting symptom and is seen in about 35-40% (40;41). Particularly in obese women with hyperandrogenism and manifest diabetes mellitus, a rare skin lesion, acanthosis nigricans, can occur (42). This symmetric hyperpigmented and hyperkeratotic thickening which is often seen like as a darkened necklace in the back of the neck, is often seen as a sign of insulin resistance (43-53).

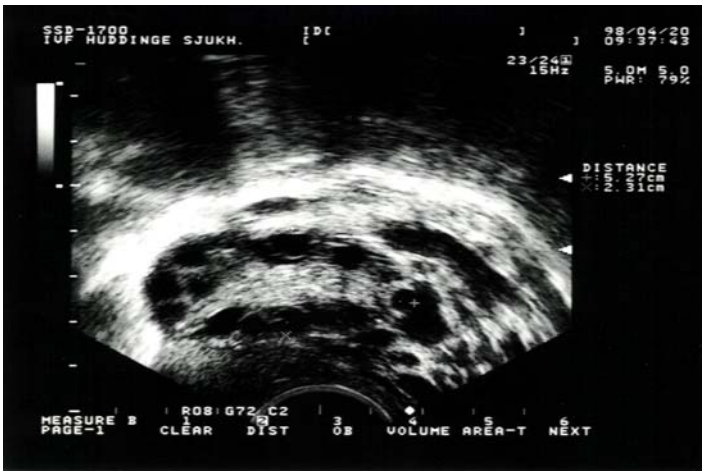


**Fig 1.** Typical ultrasound picture of PCOS ovary. (foto Ingvar Ek)

## 1.3 DIFFERENTIAL DIAGNOSIS

The differential diagnosis of polycystic ovary syndrome includes patients with menstrual disturbances and hirsutism in which the primary diagnosis is of pituitary or adrenal diseases *i.e.* hyperprolactinemi, acromegaly, and classic or nonclassic congenital adrenal hyperplasia, Cushing's disease and androgen secreting tumors (41;54). These can be identified by the presence of other specific clinical and biochemical features, and further modern radiologic investigations. Adams et al described multifollicular ovaries (MFO) 1985, as normal in size or slightly enlarged and filled by six or more cysts 4-10 mm in diameter. In contrast to women with polycystic ovaries (PCO), stroma was not increased. Unlike PCO patients, women with MFO were not hirsute and serum concentrations of luteinizing hormone and follicle stimulating hormone were normal. MFO was seen in weight loss related amenorrhea, and ovarian morphology was restored after treatment with gonadotrophin releasing hormone (LHRH) and induced ovulation in 83% of the cases. (26)

## 1.4 PATHOGENESIS

The clinical heterogeneity of the syndrome makes a polygenetic etiology more likely than a single cause. A number of endocrine abnormalities perpetuate themselves in what has been described as a "vicious cycle" (54). Abnormal gonadotrophin secretion, with high circulating LH and low, tonic FSH levels; hypersecretion by ovarian theca and stromal compartments of androgens, which were viewed as both disrupting follicular maturation and providing substrate for peripheral aromatization to estrogens in adipose and other tissues. Tonic estrogen production would lead to negative feedback on the pituitary to decrease FSH secretion and the trophic support of the granulosa cell. This concept was further supported by studies suggesting that normal ovulatory function can be restored after disruption of this cycle *e.g.*, by ovarian wedge resection or cautery or during recovery from GnRH-a induced suppression (55-58). However "the vicious cycle" concept did not explained how the disorders became established. Abnormalities in LH-secretory pattern and its regulation have been observed in PCOS, and they often have both increased LH pulse amplitude and frequency, compared with ovulatory controls (59-62). This results in increased or disordered LH secretion and may lead to an elevated serum LH:FSH ratio. However, after GnRH-a suppression no difference was seen between PCOS and normal women in the recovery of LH pulse frequency, suggesting another cause than PCOS as being primary (63). No other consistent disturbances in neuroendocrine modulators, such as the endogenous opioids, dopamine, noradrenaline, serotonin, and leptin, have been found as proposed determinants of gonadotrophin secretion in PCOS. Endogenous opioid excess may sensitize the gonadotrope to GnRH, particularly in association with hyperinsulinemia (64;65). Decreased dopaminergic inhibition of LH release (66) and an increased incidence of an allelic form of the D3 dopamine receptor have been noted in women with PCOS (67). A possible role of leptin as a factor in pathogenesis in PCOS has shown divergent results (68-72) and it is still unclear whether leptin plays a role in the etiology of PCOS or not. An intrinsic ovarian functional defect has also been postulated as the source of the self-sustaining abnormalities in PCOS. Thecal

hypertrophy and overproduction of androgens are well known features of PCOS ovary. When placed in culture, PCOS thecal cells continues to hypersecrete androgens, and when deprived of trophic support through GnRH-a suppression, the PCOS ovary continues to hypersecrete 17-OHP in response to hCG *in vivo* (73-75). Short term GnRH-a testing in PCOS produces an exaggerated ovarian 17-OHP-secretory response (73;76;77). This response may reflect the increased thecal mass present in the ovary, but has been also interpreted as reflecting dysregulation of the activity of the steroidogenic enzyme $P_{450}c17$, which is responsible for both 17-hydroxylation of C21 steroids and for the 17,20-lyase activity necessary for androgen (C19) synthesis (78). Hyperinsulinemia, secondary to insulin resistance may induce hyperandrogenism through hormonally regulated serine phosphorylation of human P450c17, suggesting a possible mechanism for human adrenarche and may be a unifying etiologic link between the hyperandrogenism and insulin resistance that characterize the polycystic ovary syndrome (79). Excessive serine phosphorylation of the insulin receptor has been proposed as a cause of peripheral insulin resistance in women with PCOS (80). Aromatase activity is low in PCOS granulosa cells *in vivo,* due to decreased FSH activity, but normal or exaggerated when they are cultured (81;82), observations that led to the concept that PCOS follicle contains excessive amounts of inhibitor(s) of FSH action. While IGFBP-2 and IGFBP-4 are FSH antagonists (83;84) that are abundant in FF from PCOS antral follicles, their expression in the PCOS ovary did not discriminate from normal cycling ovary (85-87), which is an argument against an etiological role of these proteins in this respect. Inhibin has been investigated by several authors. Antagonistic effects of LH and obesity on inhibin B production in patients with PCOS have been found (88) and Welt and co-workers results suggest that both increased LH and insulin may account for the relative suppression of inhibin B in patients with PCOS speaking for insulin as a role in this matter (89). Activin promotes ovarian follicular development, inhibits androgen production and increases FSH and insulin secretion. Eldar-Geva et al stated recently that serum follistatin is increased in PCOS patients, regardless of obesity, and that PCOS is the most significant variable that relates to high follistatin and low activin A serum concentration. A high follistatin/activin ratio could well contribute to the pathophysiology of PCOS (90). Granulosa cell mitosis also appears defective, in that granulosa cell numbers in PCOS follicles are lower than in healthy size-matched follicles from cycling women (91), but whether abnormal granulosa cell mitosis is important in the pathogenesis of PCOS has not been directly tested. Franks et al. recently found an explanation for how hypersecretion of both insulin and LH could prematurely lead to terminal differentiation of the medium sized antral follicles earlier, thought as arrested (92). Many women with PCOS develop irregular menses shortly after menarche. It has been hypothesized that excessive production of adrenal androgens, which increases at puberty, can supply substrate for extragonadal aromatization and result in tonic estrogen inhibition of FSH secretion (54). Premature adrenarche is associated with a higher incidence of both functional ovarian hyperandrogenism, with exaggerated 17-OHP response to GnRH-a challenge test (93;94) and insulin resistance (95;96). Hyperinsulinemia can stimulate adrenal as well as ovarian steroidogenesis (97). Since insulin resistance accompanies puberty and may contribute to adrenarche, an important unanswered question is why pubertal insulin resistance fails to resolve in adolescent girls who develop PCOS, and whether

the effect of hyperinsulinemia on adrenal, on ovary, or both of these organs is significant in the pathogenesis of PCOS (11).

## 1.5 INSULIN RESISTANCE IN PCOS

### 1.5.1 Introduction

**Insulin**, a pancreatic, 5900 mol wt, polypeptide hormone produced in the β-cells of the islets of Langerhans. It plays a major role in the regulation of carbohydrate, fat and protein metabolism (98). Insulin acts on its cell surface receptor (99-101). The human gene is located on chromosome 11 and encodes pre-proinsulin, a 110-amino acid single-chain polypeptide that is the precursor of insulin (98). Pre-proinsulin is proteolytically converted to proinsulin, which consists of the A chain, B chain, and C peptide. Proinsulin is homologous with IGF-I and IGF-II and can bind to the insulin receptor with approximately 10% of the affinity of insulin. Insulin is produced after the C-peptide is cleaved from proinsulin by endopeptidases active in the Golgi apparatus and in secretory granules.

**The insulin receptor** is a heterotetramer made up of two α,β-dimers linked by disulfide bonds (102). Each α,β-dimer is the product of one gene located on the short arm of chromosome 19 (103;104). The α-subunit is extracellular and contains the ligand-binding domain whereas the β-subunit spans the membrane, and the cytoplasmic portion contains intrinsic protein tyrosine kinase activity, which is activated further by ligand-mediated autophosphorylation on specific tyrosine residues (105). The insulin receptor belongs to a family of protein tyrosine kinase receptors that includes the IGF-I receptor, with which it shares substantial sequence and structural homology, as well as the EGF, fibroblast growth factor, platelet-derived growth factor, and colony-stimulating factor-1 receptors (106). After insulin binds to the α-subunit, the β-subunits become phosphorylated on tyrosine residues and acquire kinase activity, initiating a cascade of intracellular protein phosphorylation (100;105). The most important intracellular proteins phosphorylated under the influence of insulin-receptor tyrosine kinase are the insulin receptor substrates (IRSs)1-4, several of which have been described (107-115). IRS-1 appears to be important in insulin receptor function and its variant forms are sometimes associated with diabetes. Some IRS-1 mutations are associated with insulin resistance and hyperinsulinemia (116) and codon 972 polymorphism of the IRS-1 gene is associated with impaired glucose tolerance and PCOS (117). IRS-1 binds phosphatidylinositol-3-kinase (PI-3 kinase) necessary for initiation of glucose transport. In addition to PI-3 kinase activation, mitogen-activated protein kinase (MAPK) is also phosphorylated after insulin receptor binding (118) thought to be responsible for the growth-promoting effects of insulin. An alternative signaling pathway for the insulin receptor has also been described. It involves generation of inositol-glycan second messengers at the cell membrane after insulin binding to receptor α-subunits but independently of β-subunit tyrosine kinase activation (119). This alternative pathway for receptor signaling may mediate some of insulin's effects including stimulation of steroidogenesis (120-122). One of the most important insulin effects is the stimulation of the transmembrane glucose transport mediated via activated PI3-kinase. This transport is carried out by a family of glucose transporter

proteins (GLUTs), mainly GLUT4 (123) which in their resting phase, reside in intracellular vesicles, and when being activated are inserted into the plasma membrane and become functional. PI3-kinase also results in the activation of Akt (also known as protein kinase B), which in turn phosphorylates and inactivates glycogen synthas kinase-3 (GSK-3). Inactivation of GSK-3 results in activation of glycogen synthas and glycogen synthesis (124).

### 1.5.2 Insulin and insulin receptors in the ovary

Both in humans and in animal models, insulin receptors are widely distributed throughout all ovarian compartments, including granulosa, thecal, and stromal tissues (125-131). Ovarian insulin receptors have the same heterotetrameric $\alpha_2$ $\beta_2$ structure as insulin receptors in other organs. They possess tyrosine kinase activity (126) and may stimulate the generation of inositolglycans (121). As in other organs, insulin itself play a major role in the regulation of insulin receptor expression: *in vitro,* insulin exposure leads to downregulation of its own receptor, followed by a return to normal receptor number approximately 4 h after insulin exposure ends (132). *In vivo,* downregulation of ovarian insulin receptors by insulin has been observed in rats with experimentally induced hyperinsulinemia (133). In postmenopausal women, *in vivo* studies have demonstrated a positive correlation between insulin receptor number on circulating white cells and in the ovary (134). This relation was not found in premenopausal women, where other circulating factors such as gonadotrophins, or sex steroids, or locally produced autocrine regulators such as IGFs and IGFBPs, may be involved in insulin receptor regulation. These factors may account for the observation that in premenopausal women with PCOS and other hyperinsulinemic states, ovarian insulin receptor expression is preserved (86;128;135) and that the insulin receptor may mediate some of the ovarian effects of insulin despite the presence of peripheral insulin resistance (121;136;137). (Table 1)

**Table I** *Actions of insulin on the ovary which have been demonstrated both in vitro and in vivo with no significant differences between humans and other species*(127)

| *Insulin effects* | *Organ* |
|---|---|
| ***Directly stimulates steroidogenesis*** | Ovary |
| ***Acts synergistically with LH and FSH to stimulate steroidogenesis*** | Ovary |
| ***Stimulates 17 α-hydroxylase*** | Ovary |
| ***Stimulates or inhibits aromatase*** | Ovary, Adipose tissue |
| ***Up-regulates LH receptors*** | Ovary |
| ***Promotes ovarian growth and cyst formation synergistically with LH/hCG*** | Ovary |
| ***Down-regulates insulin receptors*** | Ovary |
| ***Up-regulates type I IGF receptors or hybrid insulin / type I IGF receptors*** | Ovary |
| ***Inhibits IGFBP-1 production*** | Ovary, liver |
| ***Potentiates the effect of GnRH on LH and FSH*** | Hypothalamus, pituitary |
| ***Inhibits SHBG production*** | Liver |

### 1.5.3 Insulin and insulin resistance in PCOS

Insulin resistance can be determined by measuring insulin levels during frequently sampled IVGTT (138) or by euglycemic, hyperinsulinemic clamp (HIEC) (139) or by insulin tolerance test (ITT) (140;141). A great number of women with PCOS demonstrate peripheral insulin resistance involving skeletal muscle and adipose tissue, which results in compensatory hyperinsulinemia (10). Insulin resistance does not appear to involve ovarian steroidogenesis, because granulosa and theca cells from PCOS ovaries demonstrate a normal dos response to insulin in culture (136;137). Hyperinsulinemia could therefore lead to thecal androgen hypersecretion.

Obese women with PCOS are more insulin resistant than weight-matched controls (142;143) suggesting that obesity and PCOS exert independent effect on insulin resistance. Many studies, mostly North American, have found insulin resistance in lean as well as obese subjects with PCOS (62;142;144;145), while several European studies failed to find such statistical connections (146-149), which could be attributed to differences in diagnostic criteria between US and European studies.

Insulin action in PCOS has been evaluated in various tissues, such as adipocytes, muscle, and fibroblasts. Studies in adipocytes have failed to demonstrate differences between PCOS women and weight-matched controls in both insulin receptor numbers and binding affinity (150;151). The one study that reported a decreased insulin-receptor number in adipocytes of PCOS women did not show adequately control for impact of obesity on insulin binding (152). Studies in s.c. abdominal adipocytes isolated from PCOS women have demonstrated decreased insulin receptor autophosphorylation, decreased maximal rates of insulin-stimulated glucose transport, and significantly increased half-maximal doses of insulin for glucose transport, independent of obesity

(150;151). These findings suggest a post binding defect in insulin-receptor signaling. The maximal antilipolytic effect of insulin did not differ in adipocyte from obese PCOS women and weight-matched controls (153). The hypothesis behind the resistance to the action of insulin in adipocyte is the results of increased circulation of free fatty acid (FFA) levels. FFAs then act as a second signal that leads to insulin resistance in the liver and muscle, resulting in increased hepatic glucose output and decreased glucose uptake (154). Increased flux of FFA to the liver results in increased hepatic gluconeogenesis and VLDL production, in turn causing fasting hyperglycemia and atherogenic lipoprotein profile (155-157). FFA may also contribute to insulin resistance by decreasing glucose uptake and metabolism in peripheral tissues by substrate competition (158)

Cultured skin fibroblasts have been used to assess whether there were intrinsic defects in insulin action in PCOS. In one study, no difference was found regarding to insulin binding and sensitivity (159). Another study found decreased tyrosine autophosphorylation in partially purified insulin-stimulated insulin receptors in cultured fibroblasts from PCOS women. This was secondary to high basal phosphoserine autophosphorylation. Serine phosphorylation has been shown experimentally to inhibit insulin receptor signaling (160). No mutation has been detected in the coding portion of the insulin receptor gene in PCOS women with constitutive insulin receptor serine phosphorylation (80;161). The factor responsible for the serine phosphorylation, most likely a serine kinase, is extrinsic to the insulin receptor and was seen in about 50% of PCOS subjects in this study (80). Serine phosphorylation of IRS-1 appears to be the mechanism for TNF-α mediated insulin resistance. FFA can activate protein kinase C (PKC) theta. PKCs can serine-phosphorylate IRS-1 and inhibit signaling.

As seen above several molecular mechanisms for the glucose transport defect have been suggested. In one of these, abdominal adipocytes of PCOS subjects had a lower content of the GLUT-4 glucose transporter than controls (162). In another report, PCOS adipocyte insulin sensitivity could be restored by an adenosine receptor antagonist, suggesting that depletion of cellular adenosine may lead to insulin resistance (153).

The correction of insulin resistance by a thiazolidinedione, troglitazone (163;164) suggest that women with PCOS may be deficient in signal transduction through peroxisome proliferators-activated receptor -γ (PPAR-γ), the natural ligand for which appears to be a PG of the J series or an FFA (165;166).

The link between hyperinsulinemia and the increased androgen production (Table 1) observed in PCOS has been shown in a series of studies in which insulin levels were raised (167) or lowered by various methods (163;168-171) and further suggested that insulin can stimulate adrenal steroidogenesis by enhancing sensitivity to adrenocorticotropic hormone (ACTH) (97) and can increase pituitary LH release (163). These reproductive actions of insulin appear to be limited to women with PCOS and are not seen in reproductively normal women, which suggest that PCOS itself confers this susceptibility (167;168). Insulin-lowering therapies (163;172) together with weight-reduction regimens (173-176) did not only lower fasting insulin levels, but did also restored ovulatory menstrual cycles in same chronically anovulatory women with

PCOS. In many PCOS women who did not ovulate with insulin-lowering agents alone, responsiveness to the ovulation-inducing estrogen antagonist, clomiphene citrate, was enhanced (171;172). These observations suggest that insulin resistance plays an important role in the pathogenesis of anovulation in PCOS. Neither have suppression of ovarian or adrenal steroidogenesis improved insulin resistance (177-179), nor have ovarian cautery, which lowers the androgen secretion (180). Insulin in large doses can directly stimulate ovarian androgen production, most likely via spillover occupancy of the IGF-I receptor (181). In PCOS, insulin levels are only modestly elevated and are not in the range needed to activate the type 1 IGF receptor in non-ovarian tissue (182). Recent studies suggest that insulin acts via its cognate receptor rather than by spillover occupancy of the type 1 IGF receptor in PCOS (121;136) but this is still under debate.

In addition to decreased insulin sensitivity in women with PCOS, insulin secretion also appears to be abnormal, with exaggerated early insulin response to intravenous glucose which was independent from insulin resistance and obesity (148;174;183). That may indicate an increased activity of pancreatic β-cell function in women with PCOS. This was also in concordance with findings of depressed plasma glucose levels during fasting state as well as during 24 h measurements in glucose tolerant women with PCOS (184;185). Further studies about the counter regulatory response to hypoglycemia in PCOS subjects have also been carried out (186). Both obese and nonobese women with PCOS appear to have inadequate secretion for their degree of insulin resistance (187) placing them at an increased risk for the development of NIDDM (188)

Androgens do cause mild insulin resistance in women (10). Androgen administration to female –to-male transsexuals causes modest reduction in insulin sensitivity (189). Similarly, lowering circulating androgen levels pharmacologically or by blocking androgen action with receptor antagonists results in slight improvements in insulin resistance in hyperandrogenicemic women (190). The magnitude of change was not in the range of insulin resistance associated with PCOS (190). Therefore androgens may amplify but do not account for insulin resistance in adult women with PCOS. Data from nonhuman primates, however, indicate that transient intrauterine androgen exposure may cause disordered LH release, increased central adiposity, and defects in insulin secretion that are not manifested until puberty (191;192)

## 1.6 GENETICS OF PCOS

The genetic basis of PCOS is unknown. Familial clustering of cases suggests that genetic factors play an important role in its etiology. Not all women with insulin resistance have PCOS and vice versa. States of extreme insulin resistance - Type A syndrome (193) with compromised insulin receptor or Type B syndrome (194) with antiinsulin receptor antibodies are rare but exists. Familial clustering of PCOS is well-documented and is consistent with genetic susceptibility to the disorder (195). Franks and co-workers have reported that the polycystic ovary morphology clusters in PCOS families, and a segregation analysis by these investigators were consistent with the autosomal dominant mode of inheritance when premature male-pattern balding was used to define affected males (196). This theory is now not thought to be valid (197). In

a study of siblings of PCOS women, Legro et al. found that about 50% of reproductive-age sisters of PCOS subjects had hyperandrogenemia (testosterone and DHEAS) with regular menstrual cycles and fecundity. (198). A resent study of sisters to PCOS women concluded that markers of insulin resistance are associated with hyperandrogenemia rather than menstrual irregularity in the sisters of women with PCOS. Menstrual irregularity may be related to the magnitude of insulin sensitivity or insulin secretion or to other factors associated with obesity (199). Even brothers of PCOS women have been investigated and presented significantly increased DHEAS levels (200). It is unlikely that that PCOS has a simple Mendelian mode of inheritance. It is more likely a complex disease that requires the interaction of at least several genes (201). In linkage studies a cholesterol side-chain cleavage enzyme (CYP11A) and an allele of insulin gene variable number of tandem repeats (VNTR) have been reported to be linked to the polycystic ovary-male-pattern-balding phenotype (197). The strongest evidence for linkage was found for follistatin among 37 candidate genes for PCOS (202;203). Follistatin is an activin-binding protein that neutralizes activin´s biologic activity. Activin stimulates ovarian follicular development, inhibits theca cell androgen production, and increases pituitary FSH release and pancreatic β-cell insulin secretion. Follistatin and activin are widely expressed, including in the ovary, pituitary, adrenal cortex, and pancreas. An increase in the level or functional activity of follistatin might be expected to lead to an arrest of follicular maturation, increased ovarian production, and decreased circulating FSH levels, and impaired insulin secretion (202). However sequencing of the follistatin gene has failed to detect variants associated with PCOS (203). There has been no evidence for mutations in the coding portion of the insulin receptor gene in previous studies (80;161;204;205) but evidence for a linkage of a marker (1-2 cM) in the region of the insulin receptor gene has been reported (206).

# 2 FAT CELL METABOLISM

## 2.1 INTRODUCTION

In a world where food supplies are intermittent, the ability to store energy in excess of what is required for immediate use is essential for survival. Fat cells (adipocytes), residing within widely distributed adipose tissue depots, are adapted to store excess energy efficiently as triglycerides (TG) and when needed, to release stored energy as free fatty acids (FFAs) for use at other sites. This physiological system, orchestrated through endocrine and neural pathways, permits humans to survive starvation for as long as several months. However, in the presence of nutritional abundance and a sedentary lifestyle, and influenced importantly by genetic endowment, this system increases adipose energy stores and produces adverse health consequences, as obesity, insulin resistance, glucose intolerance or NIDDM, hypertension and dyslipidemia, risk factors seen in the so called "*metabolic syndrome*" or "*insulin resistance syndrome*".

## 2.2 OBESITY, DEFINITION AND MEASUREMENT

Obesity is a state of excess adipose tissue mass. Although often viewed as equivalent to increased body weight, this need not be the case, because lean, but very muscular individuals may be over weight by arbitrary standards without having increased adiposity. Body weights are distributed continuously in populations, so that a medically meaningful distinction between lean and obese is somewhat arbitrary. Obesity is therefore more effectively defined by assessing its linkage to morbidity or mortality. Although not a direct measure of adiposity, the most widely used method to gauge obesity is the *body mass index* (BMI), which is equal to weight/height$^2$ (in kg/m$^2$). Other approaches to quantifying obesity include anthropometry (skin-fold thickness), densitometry (underwater weighing), and computed tomography (CT) or magnetic resonance imaging (MRI), and electrical impedance. Using data from Metropolitan Life Tables,(207), BMIs for the midpoint of all heights and frames among both men and women range from 19 to 26 kg/m$^2$; at a similar BMI, women have more body fat than men. Based on unequivocal data of substantial morbidity, a BMI of 30 is most commonly used as a threshold for obesity in both men and women. Large scale epidemiologic studies suggest that all-cause, metabolic, and cardiovascular morbidity begin to rise (albeit at a slow rate) when BMIs are ≥25, suggesting that the cut-off for obesity should be lowered. Some authorities use the term overweight, rather than obese, to describe individuals with BMIs between 25 or 27 and 30. A BMI between 25 and 30 should be viewed as medically significant and worthy of therapeutic intervention, especially in the presence of risk factors that are influenced by adiposity, such as hypertension and glucose intolerance (207).

### 2.2.1 Fat distribution

The distribution of adipose tissue in different anatomic depots also has substantial implications for morbidity. Specifically, intraabdominal (visceral) and abdominal subcutaneous fat have more significance than subcutaneous fat present in the buttocks and lower extremities. This distinction is most easily made by determining the waist-to-

hip ratio, with a ratio >0.9 in women and >1.0 in men being abnormal. Many of the most important complications of obesity, such as insulin resistance, diabetes, hypertension, and hyperlipidemia, and hyperandrogenism in women, are linked more strongly to intraabdominal and/or upper body fat than overall adiposity. The mechanism underlying this association relate to the fact that intraabdominal adipocytes are more lipolytically active than those from other depots. Release of FFA into the portal circulation has adverse metabolic actions, especially on the liver leading to increased hepatic gluconeogenesis and VLDL production, in turn causing fasting hyperglycemia and an atherogenic lipoprotein profile. Hyperinsulinemia may be augmented by interference of excess FFA with hepatic extraction of insulin. FFA may interact with insulin receptors and cause decreased insulin sensitivity. Glucose uptake can be inhibited through the so called *Randle's cycle*. Insulin clearance is decreased, an effect that might be dependent on fatty acid oxidation and FFA is also a major substrate for hepatic TG production, and thereby, as has already been pointed out, the assembly and secretion of VLDL will increase. (208-211)

## 2.3 LIPID SYNTHESIS

The primary function of adipose tissue is to store or release free fatty acids FFA from triglycerides (TG), during surplus or starvation of nourishment. Adult human body is composed of approximately 10-15 kg of adipose tissue, which not only functions as energy storage, but also functions as insulation and hormone production. The specific cell in the adipose tissue, the largest energy reserve of human body, is the adipocyte, which consists of >95% TG. The TG content of the fat cell comes from VLDL from the liver and chylomicrons from intestines. Uptake of glucose and FFA is facilitated by lipoprotein lipase produced from the adipocytes. Glucose is taken up in the fat cell via facilitated diffusion via the action of glucose transporters mainly GLUT-4, which is stimulated by insulin (212;213). 50-70% of fat cell glucose uptake can be metabolized to lactate, which in turn can provide lactate for hepatic gluconeogenesis during fasting or promote hepatic glycogen synthesis after food ingestion (214). Glucose can also be utilized for FFA synthesis within the fat cell, but this process is much less important in humans as compared to fat cells in other species.

The uptake of FFA is dependent on the action of lipoprotein lipase (LPL), which is synthesized by the adipocytes and secreted into the capillaries, where it hydrolyzes FFA mainly from chylomicrons (from the intestines) containing TG and from VLDL, a TG rich lipoprotein emerging chiefly from the liver (215). After uptake in the fat cell, FFA are bound to specific binding protein and transferred to intracellular organelles for TG synthesis (216). The remaining glycerol outside the fat cell is metabolized in the liver. The fat cell needs to produce new glycerol for its TG production. The LPL-activity is regulated by various hormones and condition (217). For example, insulin and cortisol induce LPL-activity. Obesity is associated with elevated LPL-activity, probably through hyperinsulinemia, whereas fasting did result in decreased levels of LPL-activity. Esterification is of FFA is very efficient in the adipocyte, whereas oxidation and ketogenesis are limited (218). In a recent study on healthy females, androgens e.g. dihydro-testosterone (DHT) stimulated both LPL-activity and hormone sensitive lipase (HSL) in an opposing manner (219).

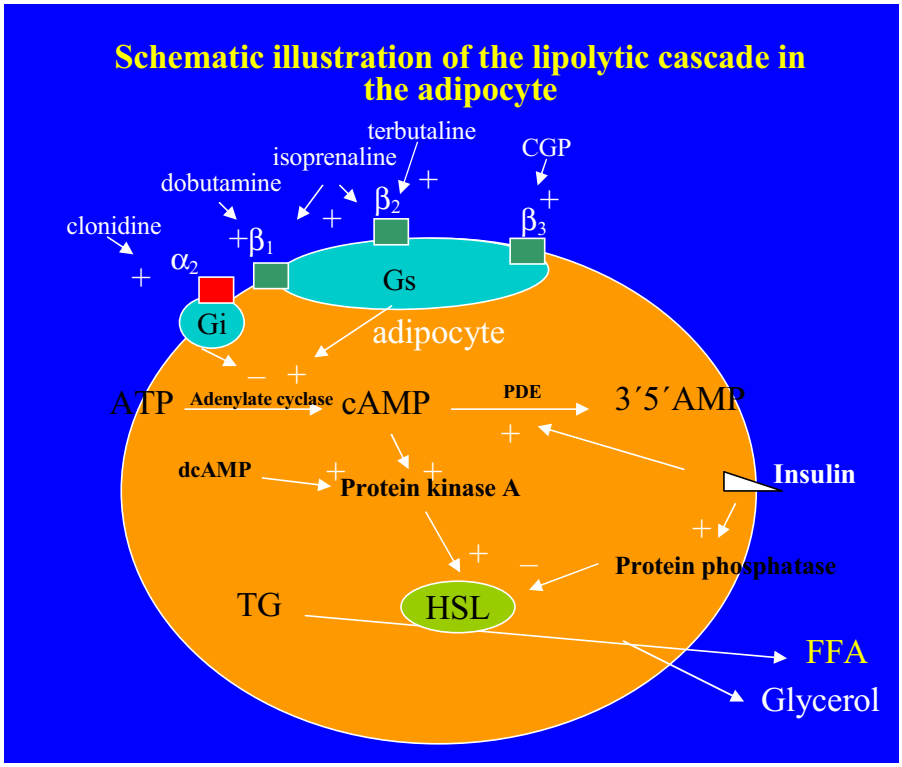


**Fig 2.** Binding of catecholamines to β-AR leads to activation of adenylate cyclase through $G_s$ protein mediated coupling. When catecholamine is binding to $\alpha_2$-AR, the $G_i$ proteins are activated causing inhibition of adenylate cyclase in turn decreases the concentration of cAMP within the fat cell. Cyclic-AMP activates the protein kinase A (PKA) complex which causes phosphorylation and activation of the hormone sensitive lipase (HSL), catalyzing the breakdown of triglycerides (TG) to fatty acids and glycerol – The final, and rate limiting step in the lipolytic cascade. Phosphodiesterase (PDE) reduces the intracellular cAMP level by breakdown to metabolically inactive 3'5'-AMP. Protein phosphatases dephosphorylate and inactivate HSL. Insulin has effects on the lipolytic cascade via binding to its own receptor by stimulating PDE and protein phophatase activity and by interactions with β-ARs

## 2.4 LIPOLYSIS

When nutritional status is in balance, lipid synthesis is counteracted by lipid breakdown (lipolysis). Lipolysis is activated in a step-wise manner, the so called *the lipolytic cascade*, (Fig 2.) whereby TG are hydrolyzed into FFA and glycerol (Figure 2). The FFA is both released to the bloodstream and transported to striated muscle and heart for oxidation, or undergoing re-esterification. The glycerol molecule is not re-esterified; instead it is always metabolized in the liver. Therefore, measurement of glycerol release is a useful indicator of lipolysis rate.

Catecholamines and insulin are the two principal hormones regulating the lipolysis process in man. Both act by binding to specific receptors localized in the adipocyte cell membrane.

Catecholamines, noradrenaline and adrenaline, either stimulate lipolysis through binding to $\beta_1$-,$\beta_2$-,$\beta_3$-adrenoceptors or inhibit lipolysis via binding to $\alpha_2$-adrenoceptors (217;220-222). The lipolysis rate in the adipocyte depends on the balance between β- and $\alpha_2$-adrenoceptors. After binding to extracellular ligandbinding site, the hormone-receptor complex stimulates the adenylate cyclase activating the formation of cAMP from ATP. cAMP activates protein kinase A which in turn causes phosphorylation and activation of HSL. The lipase catalyzes the breakdown of triglycerides (TG) to fatty acids (FFA), diglycerides, and monoglycerides – the final rate limiting step of the lipolytic cascade. Monoacylglycerollipase finally catalyzes the breakdown of monoacylglycerol to glycerol and FFA. This latter enzyme, which is abundant in adipose tissue, is not under hormone control. Phosphodiesterase (PDE) reduces the intracellular cAMP level to inactive 3´5´-AMP. Protein phosphatases dephosphorylate and inactivate HSL (223). Insulin has effects on lipolytic cascade via binding to its own receptor by stimulating PDE III (224) and protein phosphatases activity and by interaction with β-adrenoceptors (225).(Table 2).

### 2.4.1 Adenylate cyclase and G proteins in the lipolytic cascade

Catecholamine receptors belongs to a family of receptors with seven membrane spanning domains divided in 3 protein units: the ligand binding receptor, a guanyl nucleotide regulatory unit, and a catalytic unit (adenylate cyclase) (226). The regulatory unit is a coupling protein, regulated by guanine nucleotides (specifically) GTP), and therefore it is called GTP binding protein or G protein for short (227;228). The catalytic unit is the enzyme itself which converts ATP to cAMP. The receptor and nucleotide regulatory unit are structurally linked, but inactive until the hormone binds to the receptor. When binding, the complex of hormone, receptor, and nucleotide regulatory unit is activated leading to an uptake of guanosine 5´-triphosphate (GTP) by regulatory unit. This result can be viewed as the outcome of the regulatory unit *coupling* to the catalytic unit forming an intact complete enzyme. Enzyme activity is then terminated by hydrolysis of the GTP to guanosine 5´-diphosphate (GDP) returning the enzyme to its inactive state. Quick action and acute control of adenylate cyclase are assured because the G protein is a GTPas that self-activates upon binding of GTP. The ability of the hormone-receptor complex to work through a common messenger (cAMP) and produce contrasting actions (stimulation or inhibition) is thought to be due to the presence of both

**Table 2 Lipolytic active agents used in lipolysis experiments**

| Agent | Mechanism of action |
|---|---|
| ***LIPOLYSIS STIMULATORS at receptor level:*** | |
| **Noradrenaline** | Non-selective α and β-AR agonist |
| **Isoprenaline** | Non-selective β-AR agonist |
| **Dobutamine** | Selective $\beta_1$-AR agonist |
| **Terbutaline** | Selective $\beta_2$-AR agonist |
| **CGP 12177** | $\beta_3$-AR agonist, $\beta_1$/ $\beta_2$-AR antagonist |
| **Propranolol** | Selective $\beta_1$/$\beta_2$-AR antagonist |
| **ICI 118,551** | Selective $\beta_2$-AR antagonist |
| ***LIPOLYSIS INHIBITORS at receptor level:*** | |
| **UK 14304** | $\alpha_2$-AR agonist |
| **Insulin** | Binds to its own receptor Stimulating PDE and protein phosphatase<br>Interaction of β-ARs |
| **Adenosine** | Binds to its own adenosine-1 receptor |
| **$PGE_2$** | Binds to its own receptor |
| ***LIPOLYSIS STIMULATORS at post receptor level:*** | |
| **Forskolin** | Adenylate cyclase activator, (increases cAMP) |
| **Dibuturyl cAMP (dcAMP)** | cAMP analogue *resistant* to PDE (phophodiesterase) |
| **8-bromo cAMP** | cAMP analogue *sensitive* to PDE |

stimulatory and inhibitory nucleotide G proteins (229;230). However the G protein system is not limited to cAMP signal, but can also activate messenger-generating enzymes, as well as ion channels. G proteins are composed of α-, β- and γ-units of which the β- and γ-subunits are not all alike and exhibit selectivity for specific receptors *e.g.* catecholamines, FSH, LH/hCG, TSH, vasopressin, angiotensin II and dopamine. Each G protein has a unique α-subunit, and there are 16 mammalian α-subunit genes, grouped into 4 subfamilies: $G_s\alpha$, $G_q\alpha$, $G_i\alpha$, $G_{i2}$. $G_s$ and $G_q$ proteins mediate stimulatory events such as stimulation of protein kinase-A which activates HSL by phosphorylation in the lipolytic cascade, whereas $G_i$ proteins exert inhibition. The role of $G_{i2}$ is not yet certain. In the inactive state GDP is bound to the α-subunit. Hormone receptor interaction and binding change the α-subunit conformation. GTP replaces GDP on the α-subunit, freeing the β- and the γ-subunits, which allows the GTP-α-subunit to bind to the catalytic unit of adenylate cyclase, forming the active enzyme. Intrinsic GTPas activity quickly hydrolyzes the GTP-α to GDP-α, which leads to reassociation with the β- and γ-subunits, reforming the G protein complex for further activation. The functional specificity is due to the α-subunit which differs for each G protein, and therefore there are many different α-subunits encoded by different genes.

### 2.4.2 Desensitization – uncoupling from the G protein

Occupancy of the receptor causes a rapid agonist specific desensitization of the system, due to receptor phosphorylation, which uncouples the receptor from the G protein. Prolonged agonist stimulation causes internalization (sequestration) and true receptor loss from the cell surface i.e. down regulation.

### 2.4.3 Regional differences in lipolytic rate

The net lipolytic effect of catecholamine stimulation is balanced of the expression and function of the β- and $\alpha_2$-adrenoceptors. According to the theory of spare receptors, human fat cells express an excess of β-adrenoceptors and only a fraction of the receptor population has to be occupied to give maximum response (231). The sensitivity of fat cells to catecholamines depends on the receptor density. When the maximum lipolytic response to catecholamine stimulation is decreased in spite of normal amount of receptors, a post receptor defect in PKA-HSL complex should be considered. The balance of receptor type and concentrations may be important for differences in various adipose tissue depots. It is well established through *in vivo* as well as *in vitro* studies that there are regional variations in the lipolytic activity of human adipose tissue. The rate of lipolysis is low in the subcutaneous femoral/gluteal region, intermediate in the subcutaneous abdominal region, and high in the visceral/intra abdominal adipose tissue. (232). The lipolytic $\beta_1$-,$\beta_2$- and $\beta_3$-adrenoceptors are most active in the visceral fat cells, whereas the antilipolytic insulin receptors, $\alpha_2$-adrenoceptors and adenosine receptors are most active in the subcutaneous fat cells. There are important species differences in the hormonal regulation of lipolysis; only insulin and catecholamines have marked acute effects on lipolysis in fat cells of adult man. Insulin inhibits lipolysis whereas catecholamines have dual effects on lipolysis; stimulation through the different β-adrenoceptor subtypes and inhibition through $\alpha_2$-adrenoceptors (222). Antilipolytic

parahormones such as adenosine and prostaglandins, which are produced locally, are also of importance for the regulation of lipolysis in human adipose tissue.

### 2.4.4 Lipolysis in "the metabolic syndrome"

Results from several studies suggest that lipolysis is disturbed in different ways in visceral *vs.* subcutaneous fat cells in subjects with upper body obesity. Lipolytic catecholamine resistance is observed in abdominal subcutaneous fat cells of obese males due to an increased $\alpha_2$-adrenoceptor response (233). In elderly men with overt sign of the metabolic syndrome, Reynisdottir and co-workers found marked lipolytic resistance to catecholamines in isolated abdominal subcutaneous adipocytes due to a combination of decreased $\beta_2$-adrenoceptor expression and reduced ability of cAMP to activate hormone sensitive lipase (234). In contrast, increased catecholamine induced lipolysis has been observed in visceral fat cells of subjects with upper-body obesity and signs of the metabolic syndrome (235), due to increased $\beta_3$-, and decreased $\alpha_2$-adrenoceptor sensitivity. This promotes a higher rate of visceral, than subcutaneous lipolysis in these subjects resulting in increased flux of FFA to the liver via the portal venous system, contributing to the metabolic abnormalities described above (2;21).

# 3 AIMS OF THE STUDY

1. To obtain insight into possible regulatory effects of androgens on the lipolytic process by comparing adrenergic regulation in the subcutaneous abdominal adipocytes of nonobese women with PCOS and in an age- and weight matched healthy women and to study the effects on lipolysis in the former group by changing androgen status by treatment with combined oral contraceptives (**Paper I**)

2. To investigate the mechanism for impaired catecholamine-induced lipolysis in subcutaneous abdominal adipocytes in PCOS, focusing on adrenoceptors and post-receptor activation of the PKA-HSL level by studying subcutaneous adipocytes from otherwise healthy non-obese PCOS women and from age and BMI matched healthy controls. (**Paper II**)

3. To further investigate possible effects of endogenous sex steroids on fat metabolism, by studying the effects of downregulation of pituitary-, ovarian activity with GnRH agonist on the adrenergic lipolysis in subcutaneous abdominal adipocytes in healthy regularly menstruating women (**Paper III**).

4. To determine whether different steps in lipolysis regulation in subcutaneous abdominal adipocytes, from adrenoceptors to the hormone sensitive lipase complex, could be influenced in obese women with PCOS by altering androgen status, using two different intervention programs including weight reduction or combined oral contraceptives to gain more knowledge about how these factors interact and are regulated in women with PCOS (**Paper IV**)

5. To study lipolysis regulation in visceral adipose tissue in otherwise healthy young lean women with PCOS and in age and BMI-matched healthy controls in order to identify a possible primary lipolytic defect in PCOS. (**Paper V**)

# 4 MATERIALS AND METHODS

The women with PCOS (Papers I, II, IV) and controls (Paper I, II, IV) were consecutively recruited at the Department of Obstetrics and Gynecology and the Fertility clinic among patients seeking medical advice for infertility, hirsutism and oligomenorrhea or amenorrhea. The PCOS women in (Paper V) were from the waiting list for laparoscopic electrocautery of the ovaries. The controls in (Paper V) were patients from the waiting list for laparoscopic sterilization. The subjects in (Paper III) (healthy regularly menstruating women) were their own controls, and were recruited from the Fertility clinic. None of the participants used tobacco or had any medication for at least 3 months prior to examination. All women with regular cycles were investigated in the early follicular phase (days 2-8). Amenorrheic women were examined on a random day. Pregnancies were excluded. The study protocol was explained in detail to each participants and their consent were obtained. The study protocol received the approval of the ethics committee at Karolinska Institute.

## 4.1 DIAGNOSIS OF PCOS

In (Paper I,II,IV and V), the diagnosis was based on the ultrasound criteria by Adams 1986 (27) and oligomenorrhea and hyperandrogenism calculated as T/SHBG ratio >0,063 and an LH/FSH ratio above 1 on a minimum of two occasions during a period of 6 months before examination. In (Paper III) we investigated normal healthy menstruating women. The ultrasound examinations were performed transvaginally by one operator (Ek, I). Late onset of congenital adrenal hyperplasia was excluded in women with PCOS by a morning serum level of 17-OHP < 5 nmol/l (236).

## 4.2 ANTHROPOMETRIC MEASURES

All participants were examined at 08.00 h after an overnight fast. Waist/hip ratio (WHR) was measured and calculated, body mass index (BMI) was calculated as weight (kg) divided by height (m) squared. Obesity was defined as BMI >27 ($kg/m^2$). Blood pressure was determined after 30 min of rest in supine position as serum and plasma sample for hormone analysis, SHBG and glucose. Body fat content was measured by bioelectrical impedance using a TBF-305 body fat analyzer (Tanita Corp, Tokyo, Japan). This method shows a strong correlation to measurements with dual-X-ray absorptiometry according to the manufacturer (http://www.tanita.com).

## 4.3 SHORT INSULIN TOLERANCE TEST (ITT)

The tests were performed between 08.00 and 09.00 in the morning after an overnight fast. A superficial hand vein was used for blood sampling and for injection of insulin. The patients rested in the supine position for at least 30 min before the test. The test was then started with an intravenous bolus dose (0.1 U/kg) of human soluble insulin (Actrapid®; Novo Nordisk, Copenhagen, Denmark). Blood samples for blood glucose determinations were taken before the start of the test and then every other minute

during a time period of 22 min. Serum insulin was determined before, and 6 and 15 min after administration of the insulin. Heart rate, and symptoms and signs of hypoglycemia were recorded during the test. Linear regression was used to estimate the slope of decline in log transformed blood glucose concentration. Blood glucose values from 4 to 16 min were used for analysis. $K_{itt}$ was calculated from the formula $69.3/t_{1/2}$.

## 4.4 ANALYTICAL METHODS

All assays were performed at The Department of Clinical Chemistry, Huddinge University Hospital, except for 4-androstene-3,17-dione, dihydroepiandrosterone and insulin like growth factor I, for which analyses were performed at the Hormone Laboratory, Department of Obstetrics and Gynecology, Huddinge University Hospital.

Serum was used for all endocrine assays except for catecholamines. Follicle-stimulating hormone (FSH), luteinizing hormone (LH), prolactin (PRL) and dihydroepiandrosterone sulphate (DHEAS) were determined by chemiluminiscence enzyme immunoassay using commercial kits obtained from Diagnostic Products Corp., Los Angeles, CA (Immulite®). Values of FSH and LH are expressed as IU/L and PRL are expressed as µg/L of 2:nd IRP FSH 78/549 and 1:st IRP LH 68/40 and µg/L of 3:rd International Standard for Prolactin 84/50 respectively. Estradiol-17β ($E_2$), testosterone (T), 17α-hydroxy progesterone (17OHP) and insulin were determined by radioimmunoassay using commercial kits (ESTR-US-CT) obtained from CIS Bio International, Gif-sur-Yvette, France ($E_2$: ESTR-US-CT; 17OHP: OHP-CT) and Diagnostics Products Corp., Los Angeles, CA (T; "Coat-a-Count® Testosterone") and from Pharmacia Diagnostics, Stockholm, Sweden (insulin) Insulin-like growth factor I (IGF-I) were determined by radioimmunoassay after acid ethanol extraction with a commercial kit from Nichols Products Corp. San Juan Capistrano CA. The levels are expressed in µg/L of the WHO first International Reference Reagent IGF-I 87/518 (1988). 4-androstene-3,17-dione (A-4) and dihydroepiandrosterone (DHEA) were determined after extraction with diethyl ether by radioimmunoassay using in house methods (237-239)

Cortisol, thyroid stimulating hormone (TSH), free triiodothyronine (fT3), free thyroxine (fT4), growth hormone (GH) and sex hormone-binding globulin (SHBG) were determined by time resolved fluorescence immunoassay (TRFIA) using commercial kits obtained from Wallac OY, Turku, Finland (Autodelfia®). Values for TSH are expressed as mU/L and GH are expressed as µg/L of respectively the WHO 2:nd IRP for hTSH 80/558 and WHO 1:rst IRP for hGH 89/505.

Plasma levels of noradrenaline and adrenaline were determined by a modified high pressure liquid chromatography technique (240;241).

In papers I, II and V the ratio between T and SHBG (T/SHBG ratio) was used as an index of biologically active T. In women and prepubertal children, this simple ratio correlates very well with values for free or non-SHBG-bound T calculated by more complicated methods (242) and in house (243) extensive experience as well as for physic chemically determined values for these two variables see Carlström and co-

workers (243). In papers III and IV apparent concentrations of free testosterone (fT) were calculated from values for total T, SHBG and a fixed albumin concentration of 40 g/L by approximation using a computer program based upon an equation system derived from the law of mass action (244).

Detection limits and within and between assay coefficients of variation were for FSH 0.1 IU/L, 8% and 8%; for LH 0.7 IU/L, 6% and 9%; for PRL 0.2µg/L, v 2.6% and 7.2%; for DHEAS 0.002 µmol/L, 8.2% and 12%; for $E_2$ 5 pmol/L and within assay coefficient of variation 13% and 3% at $E_2$ concentrations of 23 and 87 pmol/L respectively. The between assay coefficients of variation was 18% at an $E_2$ concentration of 12 pmol/L and 6% at 94 pmol/L; for 17OHP 0.1 nmol/L, 7.8% and 10%; for T 0.1 nmol/L, 6% and 10%; for insulin 14.4 pmol/L, 5.4% and 6.4%; for IGF-I 0.6µg/L, 6% and 10%; for A-4 0.6 nmol/L,6% and 10%; for DHEA 1.6 nmol/L, 5% and 7%; for cortisol 15 nmol/L, 1.1% and 2.9%; for TSH 0.005 mU/L, 3% and 5%; for fT3 2pmol/L, 9% and 5%; for fT4 2 pmol/L, 5% and 4%; for GH 0.012 µg/L, 2,0% and 3,3% and for SHBG 0.5nmol/L, 5% and 6% respectively. For plasma noradrenaline and adrenaline the total coefficient of variation was 18% at concentration of about 2,5 nmol/L.

Plasma total cholesterol, HDL cholesterol and triglycerides and blood glucose were determined by established routine methods.

## 4.5 ISOLATION OF FAT CELLS

After an overnight fast a sc needle aspiration biopsy of adipose tissue (~ 3g) (Paper I-IV) was obtained during local anesthesia (245) from the abdominal region randomly from the left or right side at the middle of umbilicus. In (Paper V) general anesthesia using propofol in combination with fentanyl and midazolam was induced at 8:00 A.M. after an overnight fast and maintained by propofol and a mixture of oxygen and nitrous oxide. Intravenous saline was given prior to the biopsies, which were taken by laparoscopic surgery technique from abdominal omental adipose tissue before the ovarian electro cautery was performed.

Adipose tissue biopsies were immediately brought to the laboratory in saline at 37°C and the fat cells were isolated by Rodbell's method of collagenase treatment (246). First pieces of intact tissue (about 150 mg) were frozen in liquid nitrogen for later future use. The remaining specimens were cut in small (5-10 mg) fragments. Adipocytes were isolated from stroma cells by incubation with 0.5 g/L collagenase for 60 min in 5 ml of Krebs-Ringer phosphate (KRP) buffer (pH 7.4) containing 40 g/L of dialyzed bovine serum albumin at 37°C during gentle shaking. The cells were then filtered and washed three times through a silk cloth with KRP-buffer including 10 g/L of albumin and then resuspended in appropriate buffer for lipolysis (Paper I-V) and radioligand binding experiments (Paper I). This procedure eliminates stromal and vascular cells as well as remnants of hormones from interstitial fluid.

## 4.6 DETERMINATION OF FAT CELL SIZE AND NUMBERS

The cells in the albumin solution were kept constant in cell density by slow stirring. Microscopic determination of the fat cell diameter was performed using 200 cells from each subject according to DiGirolamo et al (247). The mean fat cell volume and weight were determined, taking into account the uneven distribution of the cell diameter (248). The total lipid content in each of all incubations was determined gravimetrically after organic extraction. Since the content of the spherical shaped adipocytes constitutes of >95% TG, the fat cell weight and number can be calculated by dividing the total lipid weight by the mean cell weight. The reproducibility of this method is high with a coefficient of variation of less than 3%. It has been compared with a tedious direct method by Kather et al (249), were all cells are counted in appropriately diluted cell suspensions. The two methods gave almost identical results in 10 consecutive experiments (r =0.97, linear regression analysis).

## 4.7 LIPOLYSIS EXPERIMENTS

Isolated fat cells were incubated in duplicate with air as the gas phase for 2h at 37°C, as a dilute suspension (5,000-10,000 cells/ml) in 0.2ml KRP buffer (pH 7.4), supplemented with glucose (1 g/L), ascorbic acid (0.1 g/L), and bovine serum albumin (20 g/L), with or without increasing concentrations of agents acting on different levels of the lipolytic cascade. The various agents, shown in (Table 2) were added simultaneously at the start of the incubation. The glycerol release into the medium was used as an index of lipolysis. At the end of the incubation the reaction was arrested by transferring the test tubes on ice. An aliquot (50 µL) was removed for determination of the glycerol concentration using a bioluminescence method (250). The coefficient of variation for glycerol release in duplicate samples was 4-5%. The fat cells are viable and have a linear rate of lipolysis for at least 4 h under these conditions. $\alpha_2$-adrenoceptors may be influenced antilipolytically by adenosine leaking out from isolated adipocytes (251). Therefore, in all experiments with clonidine/UK 14304 adenosine deaminase (ADA) was added to the medium to remove traces of endogenous adenosine produced during incubation. However, the influence of adenosine on lipolysis is negligible when adipocytes are stimulated with lipolytic agents (252) and was omitted in such experiments.

In antilipolytic experiments on visceral adipocytes (Paper V), the basal lipolysis rate is too low to be inhibited. In such experiments, the incubation medium was supplemented with 0.001 mol/L of 8-bromo cAMP to increase the initial (basal) lipolysis rate.

## 4.8 EXPRESSION OF LIPOLYSIS RATE

The lipolytic experiment gives information of basal lipolytic rate as well as maximum lipolytic capacity of the stimulated adipocytes. The *sensitivity* of the adipocytes to a certain agonist, defined as the concentration of the agonist giving a half-maximum effect (EC50) expressed as the agonist concentration in (mol/L), also as $pD_2$ (-log mol/L for EC50) and was calculated from logistic conversion of the dose-response curves, as described by Östman et al (253). The lower concentration of the agonist

causing half maximum effect or the higher value of $pD_2$, the more sensitive glycerol release answer to the used stimulating agonist. The maximum lipolytic response minus the basal lipolysis rate is called the agonist's *responsiveness* i.e. the delta-glycerol releases, correcting differences in the basal glycerol release between subjects. To better illustrate differences in sensitivity, the dose-response curves were plotted graphically as percent of maximal lipolysis. When maximal lipolytic response was graphically depicted, it was related to fat cell number (μmol glycerol/$10^7$ cells).

## 4.9 RADIOLIGAND BINDING ASSAY

Receptor binding studies have been described in detail previously (254). Isolated fat cells (20,000 cells /mL) were incubated at 37°C in 0.5 mL KRP buffer (pH 7.4) containing albumin (5 g/L) glucose (1 g/L), and ascorbic acid (0.1 g/L). Saturation experiments were performed to determine the total amount of β-AR´s. The cells were incubated in duplicate for 60 min with six different concentrations of [$^{125}$I] cyanopindolol ([$^{125}$I] CYP). Nonspecific binding determined in the presence of 0.1 μmol/L propranolol was about 30% at low and about 45% at high Radioligand concentrations. Competition experiments were performed in duplicate to determine the fraction of $\beta_2$-AR´s of the total β-receptor population; 100 pmol/L ([$^{125}$I] CYP) competed with 12 increasing concentrations of the $\beta_2$-specific antagonist ICI 118,551 ($10^{-11}$ – $10^{-4}$ mol/L. Nonspecific binding at $10^{-4}$ mol/L ICI 118,551 was about 30% and did not differ from nonspecific determined by 0.1 μmol/L propranolol. The binding experiments were evaluated by computerized curve fitting (Ligand, Biosoft, Furgeson, MO) (255). The software calculate estimates of the maximum total binding capacity obtained from the saturation binding experiments as well as the affinity constants ($K_d$) and the proportion of $\beta_1$-, and $\beta_2$-AR´s accessed from the displacing experiments by ICI 118,551. At the concentrations of ([$^{125}$I] CYP) used in these experiments, there was no significant binding to $\beta_3$-AR´s. Instead, the Radioligand bound with homogeneity to $\beta_1$- and $\beta_2$-AR´s, yielding linear Scatchard curves with slopes close to 1.

## 4.10 PROTEIN ISOLATION AND WESTERN BLOT ANALYSIS

(Paper II and V). Frozen tissue, ~300mg, was crushed and lysed in protein lysis buffer (1% Triton-X100, Tris-HCL pH 7.6, and 150 mmol/L NaCl, 4°C), supplemented with protease inhibitors (1 mmol/L PMSF [phenylmethylsulfonyl fluoride] and Complete;Boehringer Mannheim, Mannheim, Germany), and homogenized using a microtome. The homogenate was centrifuged at 14,000 rpm for 30 min, and the infranatant was removed and saved. The protein content in each sample was determined using a kit of reagents from Pierce Biotech (Rockford, IL). One hundred micrograms of total protein was then loaded on polyacrylamide gels and separated by standard SDS-PAGE. Samples from PCOS and control subjects were run on the same gels and transferred to the same PVDF (polyvinylidine fluoride) membranes (Amersham Pharmacia Biotech, Little Chafford, U.K.). Blots were blocked for 1 h at room temperature in Tris-buffered saline with 0.1% Tween-20 and 5% nonfat dried milk. This was followed by an overnight incubation at 4°C in the presence of antibodies directed against either the HSL complex, the catalytic region of PKA (PKAcat, 1:1,000), the regulatory regions of PKA Iα and IIβ (PKAreg Iα and PKAreg IIβ,

1:1,000), and finally against all subforms of regulatory region I (PKAreg I, 1:1,000). All primary antibodies, except those against HSL, were from Transduction Laboratories (Lexington, KY). To confirm antibody specificity, positive controls were included in all experiments as provided by the manufacturer. HSL antibodies were generated by one of the authors (C.H.; Paper V), as described below. Secondary antibodies conjugated to horseradish peroxidase were from Sigma (St. Louis, MO) (α-mouse 1:5,000, α-rabbit 1:4,000, and α-chicken 1:2,500). Antigen-antibody complexes were detected by chemiluminescence's using a kit of reagents from Pierre (Supersignal; Rockford, Rockford, IL), and blots were exposed to high performance chemiluminescence film (Amersham, Little Chalfont, U.K.). Films were scanned, and the optical density (OD) of each specific band was analyzed using the Image program (National Institutes of Health, Bethesda, MD) and expressed as ($OD \cdot mm^{-2} \cdot 100\ \mu g^{-1}$ of total protein).

## 4.11 HSL ANTIBODIES

Human HSL exists in two forms due to alternative splicing of exon 6 (256). These are commonly referred to as HSL-long and –short. In this study we used an antibody that is specific for the long variant and another that recognizes both forms. The HSL-long antibody was generated in rabbits, as previously described (256). A 15-residue synthetic peptide (QPAASPSRLLSLMDBP), Derived from the amino acid sequence encoded by exon 6, was coupled to keyhole limpet hemocyanin via an added COOH-terminal cystein residue, and was used to immunize the rabbits. The antiserum was shown to specifically recognize full-length human HSL (HSL-long), whereas it did not recognize the splice variant lacking exon 6 (HSL-short). Antibodies recognizing both HSL-long and –short were generated in chicken as previously described (257;258). In short immunized chicken antiserum was affinity-purified against recombinant rat HSL coupled to a CNBr-activate Sepharose 4B column (Amersham Pharmacia Biotech, Uppsala, Sweden). The affinity-purified antibodies were shown to be specific for both forms of HSL.

## 4.12 GENERAL STATISTICS

Student's two-tailed *t* test was used for comparison of data between (unpaired), and within (paired) groups. Data were also analyzed by ANOVA, taking into account age or fat cell volume by covariance analysis and drug concentration by repeated measure analysis. For variables not normally distributed, Wilcoxon´s signed rank test (paired observations) and Wilcoxon´s rank-sum test (unpaired observations) was used, except for $K_d$ and EC50 data which normalized by transformation into their logarithmic form before statistical analysis with parametric methods. All statistical calculations were performed with a statistical software package, (SPSS, Inc., Chicago, IL) and (Statistica, StatSoft, Tulsa, OK).

# 5 STUDY DESIGNS

## 5.1 PAPER I

*Objectives:* **1.** To obtain insight into possible regulatory effects of androgens on the lipolytic process by comparing adrenergic regulation in the subcutaneous abdominal adipocytes of nonobese women with PCOS and in an age and weight-matched healthy women, and **2.** to study the effects on lipolysis only in the PCOS group by changing androgen status by treatment with combined oral contraceptives for three months.

*Design:* Prospective, case control study

*Subjects:* Ten nonobese PCOS subjects were recruited, with clinical characteristics (mean ± SD): Age 30±3 (yr), BMI 22.6±2.2 ($kg/m^2$), WHR 0.899±0.055, S-SHBG 21±6 (nmol/L) T/SHBG 0.09±0.034. Obesity was defined as a BMI >27 ($kg/m^2$). Diagnosis of PCOS was based on clinical findings of infertility, hirsutism, oligomenorrhea/amenorrhea, typical ovarian images in ultrasound (27). Transvaginal ultrasound and androgen measurement were done in two occasions during a period of sex months prior to study and confirmed polycystic ovaries and hyperandrogenism as T/SHBG >0.063, (243) and oligo-, amenorrhea. Ultrasound technique was performed by one of the authors (I.E.). 11 controls participated and were investigated in the same way with ultrasound and serum sample excluding signs of PCOS. Clinical characteristics of controls: (mean ± SD): Age 30±8 (yr), BMI 23.1±2.6 ($kg/m^2$), WHR 0.845±0.062, S-SHBG 71±26 (nmol/L) T/SHBG 0.023±0.013.

*Adipose tissue biopsy:* A sc fat biopsy of adipose tissue (~3g) was taken during local anesthesia from the abdominal region, randomly from the left or right side at the umbilicus. The biopsy was transported directly to lipid laboratory for lipolysis experiment and radioligand binding experiment.

*Measurements:* f-S-insulin (mU/L), f-B-glucose, S-Cholesterol, S-TG, S-T, S-SHBG, T/SHBG, S-A-4, S-DHEA. BMI and WHR were measured, and f-S-insulin was the only measurement reflecting possible insulin resistance. Eight of the ten PCOS women volunteered to be reexamined according to the same protocol after a three months period of treatment with combined oral contraceptives. The second examination was performed in the same manner as the first one, except the biopsy was taken from the contra lateral side.

*Statistics:* Student's two tailed *t* test was used for comparison of data between (unpaired), and within groups (paired). The SD was used as a measure of dispersion of clinical characteristics data, and the SEM was used in experimental data. All statistics were analyzed by means of a standard software statistical package. Values for non-normally distributed parameters such as $K_d$ and EC50 were transformed into logarithmic form before statistical analysis.

## 5.2 PAPER II

*Objectives:* To investigate the mechanism for impaired catecholamine-induced lipolysis in subcutaneous abdominal adipocytes in PCOS, focusing on adrenoceptors and post-receptor activation of the PKA-HSL level by studying subcutaneous adipocytes from otherwise healthy non-obese PCOS women and from age and BMI matched healthy controls.

*Design:* Case control study

*Subjects:* Ten nonobese PCOS subjects were recruited, with clinical characteristics (mean ± SD): Age 30±4 (yr), BMI 22.4±2.8 ($kg/m^2$), WHR 0.80±0.07, S-SHBG 25±10 (nmol/L) T/SHBG 0.078±0.023. Obesity was defined as a BMI >27 ($kg/m^2$). Diagnosis of PCOS was based on clinical findings of infertility, hirsutism, oligomenorrhea/amenorrhea, typical PCOS findings in ultrasound (27). Transvaginal ultrasound and androgen measurement were done on two occasions during a period of sex months prior to study and confirmed polycystic ovaries and hyperandrogenism as T/SHBG >0.063, (243) and oligo-, amenorrhea. Ultrasound technique was performed by one of the authors (I.E.). 14 controls were recruited and were investigated in the same way with ultrasound and serum sample excluding signs of PCOS. Clinical characteristics of controls: (mean ± SD): Age 31±5 (yr), BMI 22.7±1.9 ($kg/m^2$), WHR 0.82±0.08, S-SHBG 96±56 (nmol/L) T/SHBG 0.020±0.026.

*Comment:* To further evaluate the found increased fat cell volume in the PCOS group compared to controls we added data from a previous study (Paper I) which had the same protocol in both selection criteria and lipolysis procedure, just to investigate that part.

*Adipose tissue biopsy:* A sc fat biopsy of adipose tissue (~3g) was taken during local anesthesia from the abdominal region, randomly from the left or right side at the umbilicus. The biopsy was transported directly to lipid laboratory for lipolysis and radioligand binding experiment. One piece (~300 mg) was frozen in liquid nitrogen and stored at -70°C for subsequent protein and Western blot analysis. In 2 controls and 3 PCOS the amount of adipose tissue was too small for lipolysis experiment.

*Measurements:* f-S-insulin (mU/L), f-B-glucose, S-Cholesterol, S-TG, S-T, S-SHBG, T/SHBG, S-A-4, S-DHEA. Body fat content (%), BMI and WHR were measured, and The so-called homeostasis model (HOMA)($mmol \cdot mU/L^2$) was used to calculate in vivo insulin sensitivity according to the formula: fasting plasma glucose (mmol/L) x fasting plasma insulin (mU/L) x $22.5^{-1}$ (259).

*Protein analysis and Western blot:* PKA subunits (PKA RIα, PKA cat, PKA RI, PKA RIIβ) and HSL isoforms (HSL-short and HSL-long)

*Statistics:* Student's two tailed *t* test unpaired was used for comparison of data between groups. The SD was used as a measure of dispersion of clinical characteristics data, and

the SEM was used in experimental data. All statistics were analyzed by means of a standard software statistical package. EC50 were transformed into logarithmic form ($pD_2$) before statistical analysis and a p-value of 0.05 or less was considered to be statistically significant.

## 5.3 PAPER III

*Objectives:* To further investigate possible effects of endogenous sex steroids on fat metabolism, by studying the effects of downregulation of pituitary-, ovarian activity with GnRH agonist on the adrenergic lipolysis in subcutaneous abdominal adipocytes in healthy regularly menstruating women.

*Design:* Prospective.

*Subjects:* Twelve endocrinologically healthy, normally menstruating women undergoing *in vitro* fertilization were recruited and investigated before and after 4 weeks of ovarian down regulation with GnRH-a. Both hormone analyses and fat biopsy were taken before and after ovarian down regulation. Some clinical characteristics were (mean ± SD): Age 34±3.5 (yr), BMI 22.5±1.4 ($kg/m^2$), WHR 0.70±0.22, SHBG 64±19 (nmol/L) fT 16 (7-29) (pmol/l). Transvaginal ultrasound was done before the study in order to exclude PCO or PAO.

*Adipose tissue biopsy:* A transcutaneous fat needle biopsy of adipose tissue (~3g) was taken during local anesthesia from the abdominal region, randomly from the left or right side at the umbilicus before and after ovarian down regulation. The biopsy was transported directly to lipid laboratory for lipolysis experiment. The second examination was performed in the same way as the first one, except the biopsy was taken from the contra lateral side.

*Measurements:* S-FSH (IU/L), S-LH (IU/L), S-$E_2$ (pmol/L), S-TSH (IU/L), S-DHEAS (µmol/L), S-Prolactin (µg/L), S-GH (µg/L), S-Cortisol (nmol/L), S-Testosterone (nmol/L), fT (pmol/L), S-SHBG (nmol/L), S-A-4 (nmol/L), S-17-OHP (nmol/L), P-Cholesterol (mmol/L), P-HDL cholesterol (mmol/L), fP-Triglycerides (mmol/L), fS-Insulin (mU/L), fB-Glucose (mmol/L), BMI and WHR were measured; ITT was performed before and after the GnRH-a treatment investigating changes of insulin resistance as $K_{itt}$-values.

*Statistics:* Student's two tailed *t* test for paired observations or Wilcoxons signed rank test was used for comparison of data within groups according to distribution. The SD was used as a measure of dispersion of clinical characteristics data, and the SEM was used in experimental data. All statistics were analyzed by means of a standard software statistical package. EC50 were transformed into logarithmic form $pD_2$ before statistical analysis.

## 5.4 PAPER IV

*Objectives:* To determine whether different steps in lipolysis regulation in subcutaneous abdominal adipocytes, from adrenoceptors to the hormone sensitive lipase complex, could be influenced in obese women with PCOS by altering androgen status, using two different intervention programs including weight reduction or combined oral contraceptives to gain more knowledge about how these factors interact and are regulated in women with PCOS

*Design:* Prospective.

*Subjects:* Twenty obese BMI >27 ($kg/m^2$) hyperandrogenic PCOS subjects participated in the study, with clinical characteristics (mean ± SD): Age 29±4 (yr), BMI 35.1±4.2 ($kg/m^2$), WHR 0.958±0.059, S-SHBG 19±10 (nmol/L) S-fT 0.019 (nmol/l). Transvaginal ultrasound and androgen measurement were done in two occasions during a period of sex months prior to study and confirmed polycystic ovaries and hyperandrogenism as T/SHBG >0.063, (243) and oligo-, amenorrhea. Ultrasound technique was performed by one of the authors (I.E.) The women were openly offered participation in different intervention programs, either a weight reduction (WR) program with a very low calorie diet (VLCD), described previously (260), or a 3-month treatment program with combined oral contraceptives in order to reduce the androgen level. Seventeen of the 20 women with PCOS adhered to the protocol and were reexamined after the period of treatment of OC therapy (n=8) or VLCD (n=9).

*Adipose tissue biopsy:* A sc fat biopsy of adipose tissue (~3g) was taken during local anesthesia from the abdominal region, randomly from the left or right side at the umbilicus, before and after the intervention programs. The biopsy was transported directly to lipid laboratory for lipolysis and radioligand binding experiment. One piece (~300 mg) was frozen in liquid nitrogen and stored at -70°C for future analysis. The second examination was performed in the same way as the first one, except the biopsy was taken from the contra lateral side.

*Measurements:* f-S-insulin (mU/L), P-glucose, P-Noradrenaline, P-Adrenaline, S-Cortisol, S-T, S-SHBG, fT. BMI and WHR were measured before and after interventions and f-S-insulin was the only measurement reflecting possible insulin resistance.

*Statistics:* Student's two tailed *t* test was used for comparison of data between (unpaired), and within groups (paired). The SD was used as a measure of dispersion of clinical characteristics data, and the SEM was used in experimental data. All statistics were analyzed by means of a standard software statistical package. Values for non-normally distributed parameters such as $K_d$ and EC50 were transformed into logarithmic form before statistical analysis. Non-normally distributed clinical data were expressed as the median and range, and appropriate nonparametric tests were used for statistical comparisons.

## 5.5 PAPER V

*Objectives:* To study lipolysis regulation in visceral adipose tissue in otherwise healthy young lean women with PCOS and in age and BMI-matched healthy controls in order to identify a possible primary lipolytic defect in PCOS.

*Design:* Case control study.

*Subjects:* Ten nonobese PCOS subjects undergoing laparoscopic ovarian electrocautery participated in the study. Clinical characteristics (mean ± SD): Age 29±3 (yr), BMI 23.1±3.2 (kg/m$^2$), WHR 0.81±0.07, S-SHBG 36±16 (nmol/L) T/SHBG 0.07±0.041. Obesity was defined as a BMI >27 (kg/m$^2$). Diagnosis of PCOS was based on clinical findings of infertility, hirsutism, oligomenorrhea/amenorrhea, typical PCOS findings in ultrasound (27) and indications for electrocautery. Transvaginal ultrasound and androgen measurement were done in two occasions during a period of 1-3 months prior to surgery. The hormone analyses and the short insulin tolerance test ITT ($K_{itt}$) was performed 2 weeks prior to operation on all subjects. Ultrasound technique was performed by one of the authors (I.E.). 13 controls participated from the laparoscopic sterilization waiting list and were investigated in the same way with ultrasound and serum sample excluding signs of PCOS prior to operation. Clinical characteristics of controls: (mean ± SD): Age 33±3 (yr), BMI 23.8±2.9 (kg/m$^2$), WHR 0.76±0.05, S-SHBG 67±28 (nmol/L) T/SHBG 0.022±0.012.

*Adipose tissue biopsy:* General anesthesia using propofol in combination with fentanyl and midazolam was induced at 8:00 A.M. after an overnight fast and maintained by propofol and a mixture of oxygen and nitrous oxide. Intravenous saline was given prior to the biopsies, which were taken by laparoscopic surgery technique from abdominal omental adipose tissue before the ovarian electrocautery/sterilization was performed. The fat biopsies were transported directly to lipid laboratory for all lipolysis experiments. One piece (~300 mg) was frozen in liquid nitrogen and stored at -70°C for subsequent Western blot analysis.

*Measurements:* S-FSH (IU/L), S-LH (IU/L), S-$E_2$ (pmol/L), S-TSH (IU/L), S-DHEAS (µmol/L), S-Prolactin (µg/L), S-GH (µg/L), S-Cortisol (nmol/L), S-Testosterone (nmol/L), S-T (pmol/L), S-SHBG (nmol/L), S-A-4 (nmol/L), S-17-OHP (nmol/L), P-Cholesterol (mmol/L), P-HDL cholesterol (mmol/L), fP-Triglycerides (mmol/L), fS-Insulin (mU/L), fB-Glucose (mmol/L), BMI and WHR were measured. *Protein analysis and Western blot:* PKA subunits (PKA RIα, PKA cat, PKA RI, PKA RIIβ) and HSL isoforms (HSL-short and HSL-long)

*Statistics:* Student's two tailed *t* test was used for comparison of data between (unpaired) and within groups (paired). The SD was used as a measure of dispersion of clinical characteristics data, and the SEM was used in experimental data. Data were also analyzed by ANOVA, taking into account age or fat cell volume by covariance analysis and drug concentration by repeated measure analysis. All statistics were analyzed by means of a standard software statistical package. EC50 were transformed into logarithmic form ($pD_2$) before statistical analysis.

# 6 RESULTS

## 6.1 PAPER I

*Clinical characteristics:* The nonobese women with PCOS showed several features of insulin resistance syndrome, such as higher WHR, fasting insulin levels, fasting blood glucose levels and triglyceride level.

*Lipolysis experiment:* There was no difference in the basal lipolysis rate between PCOS women and control subjects. The PCOS women showed marked resistance to the lipolytic effect of noradrenaline due to defects at two different levels in the lipolytic cascade: first a 7-fold reduction in sensitivity to the $\beta_2$-selective agonist terbutaline ($p<0.005$), which could be ascribe to a 50 % lower $\beta_2$-adrenoceptor density ($p<0.02$) according to the radioligand binding experiments. There was no difference with regard to dobutamine ($\beta_1$) or clonidine ($\alpha_2$-sensitivity) or $\beta_1$-adrenoceptor density; second, the maximum lipolytic response, stimulating the adipocytes with noradrenaline was also 40% lower ($p<0.05$) and 35% lower, stimulating with terbutaline, in the PCOS women compared to that in the healthy women. This lower responsiveness was seen with all β-adrenergic agonists and the post receptor-acting agents forskolin (activating adenylate cyclase) and dibutyryl cAMP (activating PKA-complex).

*Radioligand binding:* The total β-AR density was slightly lower in the PCOS group than in healthy controls, but not statistically significant. The PCOS women showed a 50% lower $\beta_2$-AR subtype density, 1.6 (amol/mm$^2$) compared to 2.9 (amol/mm$^2$) in control subjects ($p<0.02$) and there was no significant difference between the $\beta_1$-AR subtype densities in these two groups. Moreover there was no significant differences between the these two groups with regard to receptor affinity ($K_d$) for displacing drug ICI 118,551 or the radioligand, respectively.

*Hormone therapy:* Treatment with PCOS women with OC for three months significantly increased the S-SHBG level, resulting in a normalization of the free testosterone level, as judged by the T/SHBG level. There were no significant changes in fasting insulin, blood glucose, or triglyceride levels after OC therapy. The concentration response curves for all the lipolytic agents were almost superimposed, when comparing before and after OC treatment, and there were no changes in $\beta_1$-, and $\beta_2$-AR densities or binding affinities.

## 6.2 PAPER II

*Clinical characteristics:* There were no significant difference in BMI, age, WHR and body fat content of PCOS women and controls, which were respectively, 22.4±2.8 and 22.7±1.9 ($kg/m^2$); 30±4and 31±5(yr); 0.80±0.07 and 0.82±0.08; and 28±5 and 30±6 (%). Fasting circulating levels of insulin, glucose and lipids were normal in both groups, as were also their insulin sensitivity measured by HOMA index. The fat cell volume were 25% larger in the PCOS group as compared to controls ($p<0.05$). In PCOS women total serum levels of testosterone were increased and SHBG were decreased as expected.

*Lipolysis experiment:* Fat cell volume was (25%) significantly increased in PCOS women and it was still significant when pooling data from (Paper I) with 10 PCOS subjects with the same BMI and age ($p<0.05$). The rest of the lipolysis experiment was done with the present 10 PCOS subjects, but the adipose tissue was not enough in 3 PCOS subjects, and in 2 control women to perform the lipolysis experiment resulting in PCOS (n=7) and controls (n=12). There was no difference in basal lipolysis rate between the groups. The PCOS women showed a 40% decreased noradrenaline-induced lipolysis ($p<0.05$) which could be attributed to a 10-fold decreased $\beta_2$-receptor sensitivity and a low ability of cAMP to activate the PKA-HSL complex ($p<0.05$

*Protein isolation and Western blot analysis of subcutaneous adipose tissue:* ). In PCOS the adipocyte protein content of $\beta_2$-AR, HSL and the regulatory IIβ component of PKA were 70,55 and 25% decreased, respectively ($p<0.001$) but there was no change in the amount of the catalytic subunit of PKA or of $\beta_1$-ARs. Thus lipolytic catecholamine resistance of subcutaneous adipocytes in PCOS is probably due to a combination of decreased amounts of $\beta_2$-AR, the regulatory IIβ component of PKA and HSL, which could cause low *in vivo* lipolytic activity and enlarged subcutaneous fat cells, later leading to obesity in PCOS.

## 6.3 PAPER III

*Clinical characteristics:* Before GnRH-a ovarian down regulation, the women showed no sign of metabolic or hormonal aberration with normal BMI, fS-insulin, $K_{itt}$ value and early follicular phase sex steroid values. When down regulated the serum levels of LH, TSH, GH, PRL, $E_2$, T, fT, A-4 and 17-OHP significantly decreased, but P-cholesterol and P-HDL cholesterol raised significantly indicating the influence of these hormones on the metabolic state. The $K_{itt}$ value was not influenced by the treatment, indicating no change in the action of insulin. Blood pressure was also unchanged.

*Lipolysis experiment:* The lipolytic sensitivity ($pD_2$) for the endogenous catecholamine noradrenaline was significantly (8-fold) decreased after GnRH-a treatment, partly due to a 3-fold decrease in the sensitivity when stimulating the subcutaneous adipocytes with the $\beta_1$-AR subtype dobutamine. All other pre- or post receptor acting agents did not change the lipolytic sensitivity. There was no change in the lipolytic responsiveness [net glycerol release ($\mu mol/10^7$ cells/2h)] of all the lipolytic drugs. The amount of adipose tissue material was not enough to do radioligand binding experiments.

## 6.4 PAPER IV

*Clinical characteristics:* The VLCD caused a mean weight reduction of 8 ± 3 kg, whereas weights were unchanged in the OC group. The SHBG level rose significantly in both groups, but more markedly in the OC group, whereas total serum T decreased equally in both groups, resulting in a normalized fT level. There were indirect signs of improved insulin sensitivity, such as significant lower levels of fasting insulin and blood glucose in the WR group, but not in the OC group, indicating that insulin resistance was still present in the latter group. Plasma levels of catecholamines and arterial blood pressure fell significantly in the WR group, but not in the OC group, indicating a reduced sympathetic activity. The WR group were about nine years older than the OC group ($p<0.01$), but there were no other clinical differences before interventions.

*Lipolysis experiment:* WR caused a 50% reduction of basal lipolysis rate. The lipolytic sensitivity ($pD_2$) of the endogenous catecholamine noradrenaline was increased 10-fold after weight reduction ($p<0.03$), whereas treatment with OC caused an opposite 7-fold decreased sensitivity ($p<0.04$). Likewise the lipolytic sensitivity to the nonselective β-AR agonist isoprenaline increased 100-fold after WR ($p<0.03$), but decreased 70-fold after OC therapy ($p<0.05$). WR, caused a significant 8-fold increase in $\beta_2$-AR sensitivity (terbutaline; $p<0.02$), which were unchanged in the OC therapy group. The $\beta_1$-AR sensitivity differed between the groups; OC caused a 10-fold lower sensitivity ($p<0.03$), but were unchanged in the WR group. When lipolysis was stimulated at the adenylyl cyclase level with forskolin or at the level of PKA-complex with dibutyryl cAMP (dcAMP), no significant change in the maximum lipolysis was observed. There was a significant change in adipocyte size in the VLCD group, but not in the OC group.

*Radioligand binding:* WR caused a significant increase in total β-AR density, solely due to a selective increase in $\beta_2$-AR density. The $\beta_1$-AR density was unchanged. The β-AR affinity ($K_d$) was unchanged in both the groups. The OC treatment caused a slight, but insignificant lowering of the total β-AR density, evenly distributed in both the β-AR subgroups.

*Comment:* The impact on OC treatment lowering the $\beta_1$-AR sensitivity 7-fold, without a reduction on $\beta_1$-AR density and with full effect when stimulation with post receptor acting drugs, suggests that there is a partial uncoupling of $\beta_1$-ARs at the level of G-proteins.

## 6.5 PAPER V

*Clinical characteristics:* The nonobese women with PCOS had a slight but significant decrease in $K_{itt}$ indicative of some in vivo insulin resistance. However they showed no other features of the insulin resistance syndrome because WHR, fasting plasma levels of insulin, glucose, triglycerides and cholesterol levels, as well as BMI (23.1 in PCOS and 23.8 in controls) were comparable with those of control women. There was no difference between PCOS patients and the control subjects concerning the estimated subcutaneous and visceral adipose tissue, irrespective of whether the total area or the proportion of the different fat depots was determined by CT at L3 and L4 levels. Likewise there were no differences in fat cell weight between the groups. As expected from measurements of sex hormones and binding proteins, the PCOS women were hyperandrogenic. They were also 4 years younger then control women, but both groups had normal blood pressure.

*Lipolysis experiment:* Stimulation of the visceral adipocytes lipolysis with the endogenous catecholamine noradrenaline or $\beta_1$-, $\beta_2$- and $\beta_3$-AR selective agonists was much more efficient than in control women. This was also true for stimulation at the level of adenylate cyclase with forskolin or at the PKA level with dcAMP. All mean curves for lipolysis stimulating agents differed significantly when PCOS and control state were compared. There were no differences in inhibition of lipolysis at the levels of insulin receptor, $\alpha_2$-AR, adenosine receptor and the prostaglandin receptor. Basal lipolysis rate was similar in the two groups, as well as the sensitivity of any of the agonists used. The responsiveness (lipolytic rate at maximum effective concentration) of all the different stimulating drugs, representing different receptors and different levels in the lipolytic cascade, was significantly (50%) increased in the PCOS group. The responsiveness for all the antilipolytic agents, including insulin was similar in PCOS subjects and controls. There were no correlation between insulin sensitivity ($K_{itt}$) and lipolytic responsiveness for any agent ($r<0.31$).

*Protein isolation and Western blot analysis of visceral adipose tissue:* In the nonobese PCOS women we found a two-fold increased level of the catalytic and regulatory Iα component of PKA. In contrast, the regulatory RIIβ component of PKA was almost 50% reduced compared to control subjects. Recent studies on genetically modified mice have shown that a similar transition in the regulatory PKA units induces an increased lipolytic response to catecholamines (261-263). Further analysis showed that the level of HSL-short, an enzymatically inactive form of HSL, was decreased in the PCOS women.

*Comment:* This altered visceral lipolysis in PCOS is different from that found in the insulin resistant syndrome, where the changes in lipolysis occur at the level of adrenergic receptors.

# 7 DISCUSSION

## 7.1 INCLUSION CRITERIA

PCOS is indeed a heterogeneous group of women. The definition is still in debate. In the present studies, we have focused on hyperandrogenism and therefore one of the inclusion criteria were T/SHBG ratio above 0.063 to be certain that there was an adequate circulating level of free testosterone (243). That means that we in most cases got subjects with low S-SHBG value and also therefore women who probably were slightly insulin resistant and exhibit biochemical abnormalities. The rest of criteria used to define the PCOS groups were according to the ultrasound criteria mentioned before (8) (Page 2) and amenorrhea or oligomenorrhea.

## 7.2 GENERAL DISCUSSION

Polycystic ovary syndrome seems to have much in common with "the metabolic syndrome", as upper body obesity, insulin resistance, glucose intolerance, or NIDDM, hypertension, dyslipidemia, and increased risk of cardio- and cerebrovascular diseases – A major health hazard in industrialized communities (17-21;264).

Many prospective studies have shown that excess fat in upper part of the body (*i.e.* central or abdominal) considered by Vague (265) 1947 as "android" or male-type obesity, is a strong and from total obesity independent risk factor for mortality and metabolic disorders. In most of studies, the body fat distribution was assessed using simple anthropometric measurements, such as skin fold and WHR, measurements. Although the WHR is simple and provides a useful estimation of upper-body fat (266), it does not distinguish between deep abdominal (visceral) fat and subcutaneous abdominal fat depots. Imaging techniques, particularly computed tomography (CT), which clearly distinguishes fat from other tissues, allows the measurement of visceral and subcutaneous abdominal fat distribution.

Several studies have shown that the detrimental influence of abdominal obesity (visceral fat area) on metabolic processes is chiefly associated to the intraabdominal fat depot. (267-269).

Although a cause-effect relationship has not been definitively established, the available evidence indicates that visceral fat is an important link between the many facets of the metabolic syndrome: glucose intolerance, hypertension, dyslipidemia, and insulin resistance (270). Thus the PCOS is an interesting model to study early disturbances in relative young individuals to get more insight in the pathogenetic process behind "the metabolic syndrome". To remove obesity as a confounding factor, we did choose to investigate nonobese PCOS subjects (Paper I-III, V) with BMI <27 ($kg/m^2$) (mean ~23 ($kg/m^2$). In these lean PCOS subjects we found marked noradrenaline resistance in the subcutaneous adipose tissue, due to two major defects in the lipolytic cascade. In first hand a seven-fold reduced $\beta_2$-AR sensitivity due to 50% reduction in $\beta_2$-AR density and in addition we also found 35% lower responsiveness, when stimulating the lipolysis at maximum agonist concentration, both at receptor and post receptor level,

indicating reduced function in the HSL-PKA complex, the rate limiting step in the lipolytic cascade. Both these defects could promote the accumulation of fat in the subcutaneous abdominal depot. Although these subjects were lean, they had slightly larger fat cell size and higher WHR compared to controls. Lipolytic catecholamine resistance is observed in abdominal subcutaneous fat cells of obese males due to an increased $\alpha_2$-adrenoceptor response (233) In elderly men with overt sign of the metabolic syndrome, Reynisdottir and co-workers found marked lipolytic resistance to catecholamines in isolated abdominal subcutaneous adipocytes due to a combination of decreased $\beta_2$-adrenoceptor expression and reduced ability of cAMP to activate hormone sensitive lipase (234) A down regulation of lipolysis could be an early step in development of abdominal obesity and metabolic syndrome. An compensatory increased sympathetic tone could lead to insulin resistance and increased lipolysis in other fat depots as the important visceral. Indeed increased catecholamine induced lipolysis has been observed in visceral fat cells of subjects with upper-body obesity and signs of the metabolic syndrome (235), due to increased $\beta_3$-, and decreased $\alpha_2$-adrenoceptor sensitivity. This promotes a higher rate of visceral, than subcutaneous lipolysis in these subjects resulting in increased flux of FFA to the liver via the portal venous system, contributing to the metabolic abnormalities described above (2;21). The recently found $\beta_3$-ARs, functional in man, especially in omental fat (235) plays only a minor role in the regulation of lipolysis in subcutaneous adipose tissue (271), and it does not interfere with the $\beta_1$-, $\beta_2$-ARs selective agonists studies used in either of our subcutaneous studies.

When treated the PCOS subjects with OC in order to reduce the influence of androgens, there were no significant change in the basal insulin state and lipid profile indicating insulin resistance still present. It did not even affect lipolysis at all, possibly a slight but not significant increase in the density of $\beta_2$-ARs could be seen. OC treatment causes many changes in sex steroid profiles with high levels of synthetic estrogens and gestagens and an tremendous increase in many major hormone binding globulins causing not only reduction in free testosterone levels, but changes to other hormone as GH, cortisol, thyroxine as well. With this in mind and the small number of individuals in our study firm conclusion of the role of the sex steroids could not be drawn. However changes in steroid hormones, GH and insulin, may contribute to abdominal fat depot accumulation and this might cause the metabolic syndrome in susceptible individuals (208;272)

To get more insight to the mechanism behind the reduced responsiveness in the abdominal adipocytes in (Paper I) the PKA-HSL complex was further investigated (Paper II) Adipose tissue was again removed from lean PCOS women for Protein and Western blot analysis on: $\beta_1$-, $\beta_2$-ARs, HSL, the catalytic region of PKA (PKAcat) and regulatory regions of PKA (Iα and IIβ). Compared with (Paper I) the clinical characteristics were very well matched, apart from the fact that the insulin resistance now measured as HOMA index, did not differ significantly from controls. Fasting insulin levels did not differ significantly as well, but fat cell size was significantly 25% larger in PCOS subjects and when pooled with subjects from (Paper I) it was still significant. The PCOS showed also a 40% reduced noradrenaline induced lipolysis in confirming the data from our previous work (Paper I). The main findings were also decreased amounts of $\beta_2$-Ars (70%), HSL (55%) and regulatory IIβ of PKA in the

PCOS women compared to controls. The lipolytic catecholamine resistance in lean PCOS women is probably attributable to a combination of all these findings. And a novel mechanism for impaired subcutaneous lipolysis was revealed in our non-obese women with the polycystic ovary syndrome.

While there is a consensus that visceral fat has a strong association with cardiovascular risk factors, particularly dyslipidemia and hyperinsulinemia (209), the primary importance of visceral adipose tissue vis-à-vis subcutaneous abdominal obesity with regard to insulin sensitivity of glucose metabolism, has been challenged by some authors (273;274). They found that abdominal subcutaneous fat, as determined by magnetic resonance imaging and CT, was at least as strong a correlate of insulin sensitivity (evaluated by the euglycemic clamp) as visceral fat and retained independent significance after adjusting for visceral fat (274) This support that our findings could be a candidate as an early link to the insulin resistant state.

In order to further evaluate the influence of endogenous sex steroids on lipolysis, without the influence of insulin resistance and hyperandrogenemia we studied the effects of downregulation of pituitary-ovarian activity with gonadotrophin releasing hormone agonist (GnRH-a) on adrenergic lipolysis in healthy regularly menstruating women with BMI from 20-26 ($kg/m^2$). (Paper III). The $K_{itt}$ value was not influenced by the treatment indicating no change in the action of insulin. Not only serum levels of estrogens ($E_2$) and androgens as fT, A-4 and 17-OHP was decreased, but also TSH, GH, and PRL decreased significantly, probable secondary to decreased estrogen levels. P-cholesterol and P-HDL rose significantly indicating the relationship between the metabolism of lipids and the hormones changed. However even the lipolytic sensitivity ($pD_2$) for the endogenous catecholamine noradrenaline was significantly (8-fold) decreased after GnRH-a treatment, partly due to a 3-fold decrease in the sensitivity for the $\beta_1$-AR subtype dobutamine. No other lipolytic change was seen in all the used drugs at different levels of the lipolytic cascade. Unfortunate radioligand binding experiment could not be done, due to lack of adipose tissue since only small amounts of adipose tissue could be removed by needle biopsy in these lean subjects.

There are several endocrine changes induced by the ovarian down regulation with GnRH-as, that in theory could be associated with the change in lipolytic sensitivity to adrenergic stimulation. GH has previously been shown to increase the lipolytic sensitivity to catecholamines in adipocytes from healthy adults (275). At first, the decreased lipolytic sensitivity, may be due to decreased GH levels, but on the other hand, IGF-I levels were almost identical before and after GnRH-a treatment, which speaks against a role of GH in this respect. However, since there is a synergistic effect between GH and testosterone one cannot exclude a permissive effect on those hormones on the adrenergic stimulated lipolysis. There are numerous reports on stimulatory as well as inhibitory effects of androgens, notably T, on catecholamine stimulated lipolysis in various animal model systems (276;277) and the existence of androgen receptor in human adipocytes has been clearly demonstrated (278). Published data on androgens status and lipolysis in women, including our own studies, have usually included subjects who were hyperandrogenic or overweight or both. It is therefore difficult to compare these data with those obtained in this study (Paper III) on lean healthy subjects.

When obese women with or without hyperandrogenism were subjected to weight reduction, (reducing insulin resistance) circulating levels of total as well as free T decreased (Paper IV) (260) as in this study (Paper III) following ovarian down regulation. However, in contrast to (Paper I-III) results, the lipolytic sensitivity to stimulation with catecholamines increased rather than decreased, when free testosterone levels decreased in conjunction with weight reduction. It shall be kept in mind that the decreased in T and fT levels, in these groups of obese women was accompanied by a fall in circulating insulin levels, the most important endogenous lipolytic inhibitor. In (Paper III), fasting insulin levels or $K_{itt}$ values did not change. GnRH treatment in hyperandrogenic, anovulatory women have been reported with increased adiposity, an indirect sign of impaired lipolysis (279). Furthermore, one study reported positive correlation between noradrenaline stimulated lipolysis and biologically active T expressed as T/SHBG-ratio, in women with PCOS (280). Based on above mentioned findings and our own results, we suggest that androgen status may therefore be of importance for lipolysis regulation in normal healthy women as well as women with PCOS. However in the PCOS group, insulin resistance seems to override the effects of androgen status of lipolysis regulation, at least in a short term intervention perspective.

The aim of intervention study (Paper IV) was to see whether two different treatments (weight reduction with VLCD or OC treatment), known to decrease the androgen levels could influence the adrenergic lipolysis in subcutaneous adipocytes in obese PCOS women. (See page 28). WR caused a 50% reduction in basal lipolysis rate and a 5- to 7-fold increased noradrenaline and terbutaline sensitivity, the latter described as a 2-fold increase in $\beta_2$-ARs density measured with radioligand binding. There were no changes as regard to $\beta_1$-AR sensitivity or density or, $\alpha_2$-AR sensitivity in the WR group. In OC treatment we did not see any influence on basal lipolysis rate or in $\beta_2$-, or $\alpha_2$-AR sensitivity. However the $\beta_1$-AR sensitivity was lowered 7-fold without a decrease in $\beta_1$-AR density. This effect was not seen at post receptor level or at the HSL-PKA complex level, suggesting a partial uncoupling of $\beta_1$-ARs (See page 16, [2.4.2.]). WR therapy but not OC therapy caused in addition to changes in lipolysis function, improved *in vivo* insulin sensitivity and lower plasma noradrenaline levels. According to (Paper III) this suggests that androgens play a minor role in obese PCOS women and insulin plays a major role in this respect. Furthermore could disturbances in sympathetic pathways be of certain pathogenic importance. Sympatho-adrenal reflexes and the autonomous nervous system play an important role in the development of the insulin resistant (metabolic) syndrome (281).

As emphasized before, PCOS has a strong resemblance to the insulin resistance (metabolic) syndrome, were an increased rate of visceral fat cell lipolysis is believed to play a role in pathogenesis. We hypothesized that primary defects in visceral lipolysis might also exist in PCOS. To take into account obesity as a confounding factor, we studied lean PCOS women and BMI matched controls. PCOS women were recruited among those from the waiting list undergoing laparoscopic ovarian cautery and the controls from the laparoscopic sterilization waiting list. For all subjects BMI ranged from 19-27 ($kg/m^2$), and age ranged from 24-39 years. Ten non obese PCOS women were compared with 13 matched controls. In vitro lipolysis regulation and stoichiometric properties of the final step in lipolysis activation namely the protein

kinase A (PKA)-hormone sensitive lipase (HSL) complex, were investigated in isolated visceral adipocytes.

The overall visceral adipocyte lipolysis was significantly (50%) increased compared to controls. This was seen in all the receptor- as well as the post receptor stimulating drugs used in the *in vitro* experiments. No significant difference in the inhibiting drugs could be seen were the curves of insulin were almost superimposed. The *in vivo* insulin sensitivity as measured with ITT was slightly increased in the PCOS women. Western blot analyses of visceral adipose tissue showed twofold increased levels of the catalytic and regulatory Iα components of PKA. In contrast, the regulatory RIIβ components of PKA was almost 50% reduced (compared to 25% in subcutaneous adipose tissue in Paper II). Furthermore protein analyses revealed the HSL-short levels (an enzymatically inactive splice form of HSL) was decreased in the PCOS women. We concluded that PCOS women even though, they were lean, had increased visceral lipolysis and that could be caused by a unique alteration in the stoichiometric properties of the adipose PKA-HSL holoenzymes and that could be an early and possible primary lipolysis defect in PCOS.

Compared to "the metabolic syndrome" which shows an increased lipolysis rate in visceral adipocytes (208;272), in most cases, due to an increased $\beta_3$-AR subtype sensitivity to the endogenous catecholamine noradrenaline (282) we have found both similarities and dissimilarities between PCOS and the insulin resistant syndrome concerning the lipolysis both in subcutaneous adipose tissue and in visceral adipose tissue. Nevertheless both result in increased flux of free fatty acids to the portal venous system and indeed are thought to be worsened, when BMI is increasing, causing known cascades of metabolic disturbances and decreased hepatic insulin extraction and clearance, which contributes to higher basal insulin values. (283;284)

## 7.3 SUMMERY AND CONCLUSION

In nonobese women with polycystic ovary syndrome, adrenergic lipolysis was investigated in both subcutaneous abdominal and visceral adipose tissue and several, disturbances were found, not known before.

In subcutaneous adipose tissue, there was a markedly impaired lipolysis, due to decreased $\beta_2$-AR sensitivity as well as density and a novel mechanism in the PKA-HSL complex, together reducing the activation of HSL. The visceral adipose tissue lipolysis was enhanced by again stoichiometric changes in the PKA complex subunits.

These abnormalities promotes accumulation of fat in abdominal subcutaneous depot and "burn off" fat in the visceral depot, thus exposing the liver to a high FFA flux, which could contribute to dyslipidemia and hepatic insulin resistance. This is supported by anthropometric data on fat cell size and computed tomography of fat depots.

In obese subjects with PCOS, weight reduction was more effective than oral contraceptives in restoring at least in part, some defects in lipolysis in subcutaneous abdominal tissue. This indicates that in obese subjects, insulin resistance seems more important, than sex steroids in regulation of lipolysis. However in lean healthy women, ovarian downregulation, showed an impairment of catecholamine lipolysis without affecting insulin sensitivity, speaking for a complex role of sex steroids in regulation of adipose tissue lipolysis.

Further investigations are needed to clarify the relationships between the different sex steroids in the regulation of lipolysis, both in PCOS and in healthy women.

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