

METABOLIC ASPECTS OF
POLYCYSTIC OVARY SYNDROME AND OBESITY

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Metabolic Aspects of Polycystic Ovary Syndrome and Obesity

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Preface

The present thesis is based on studies carried out from March 2004 to April 2006, and describes the results of my work as a clinical research fellow at the Department of Obstetrics and Gynaecology, Copenhagen University Hospital of Hvidovre.

Polycystic ovary syndrome (PCOS) has become the most common endocrine disorder amongst young fertile women and the most common cause of female infertility. The disorder has an estimated prevalence of 5 to 10%, which may be increasing with the increasing incidence of obesity.

The pathophysiological mechanisms behind the development of PCOS are poorly understood. The disorder is associated with dysfunction of many organs such as the liver, pancreas, adrenals, ovaries, hypothalamus, pituitary, muscular and fatty tissue, but how these dysfunctions are linked to the development of the PCOS phenotype is not fully understood. Hereditary factors play a role, and an autosomal dominant inheritance has been suggested. However, the genes involved are yet to be identified.

I first gained interest for the pathophysiological mechanisms of polycystic ovary syndrome during my residency at the Department of Endocrinology at Copenhagen University, Hvidovre, where I met these

patients in the out-patient clinic. I acquainted Kirsten Nørgaard, consultant at the same department, with my interest, and she referred me to Lisbeth Nilas, consultant at the Department of Obstetrics and Gynaecology, Copenhagen University Hospital, Hvidovre, and to Sten Madsbad, Professor at the Department of Endocrinology, Copenhagen University Hospital, Hvidovre. I knew instantly that I had found the perfect co-workers to address the metabolic issues in PCOS. Lisbeth Nilas had the clinical experience with this particular group of patients and Sten Madsbad had the metabolic and methodological knowledge and the experimental facilities. Together our aim has been to investigate the pathophysiological mechanism in PCOS at a molecular-biological level in some of the relevant tissues such as fatty tissue, peripheral muscle, the adrenal glands, the pancreas and the liver. Our work has provided new knowledge about:

- The independent effect of PCOS and obesity on glucose/insulin metabolism
- Incretin hormone secretion in women with PCOS
- The effect of metformin on incretin hormone secretion in lean and obese women with PCOS
- Subcutaneous adipose expression of 11 β hydroxysteroid dehydrogenase

types 1 and 2 (11 β -HSD-1 and 2) and hexose 6 phosphate dehydrogenase (H6PDH) in women with PCOS

- Skeletal muscle mitochondrial function in women with PCOS
- The effect of rapid weight loss on glucose, insulin and lipid metabolism and androgen levels
- The effect of rapid weight loss on mitochondrial function in obese healthy women
- How the insulin resistant phenotype of PCOS is defined

There is still ongoing research and data collected in connection with this project. In collaboration with Jørgen Wojtaszewski, Jonas Møller and Bente Kiens at the Copenhagen Muscle Research Centre, we are investigating the insulin signal transduction cascade before and after metformin treatment in women with PCOS. In collaboration with Bente Kiens, we are investigating lipase activity in adipose tissue of women with PCOS.

During my research years, I have come across many extremely skilled and experienced researchers and other medical workers, many to whom I owe great thanks. First and foremost I owe great thanks to Lisbeth Nilas, who has been not only an extremely committed, honest and patient supervisor but

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Finally I would like to thank my dear husband, Nicolas, for mathematical assistance and for giving my life so much value together with our three lovely children, Johannes, Karen and Zakarias

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The present thesis is based on studies carried out during the period 2004–2006 at the Department of Obstetrics and Gynaecology and at the Department of Endocrinology, Copenhagen University Hospital, Hvidovre, Denmark.

The thesis is based on the following papers:

- I.* Svendsen PF, Nilas L, Nørgaard K, Jensen JE, Madsbad S. Obesity, body composition and metabolic disturbances in polycystic ovary syndrome. *Hum Reprod.* 2008;23:2113-21.
- II.* Svendsen PF, Nilas L, Madsbad S, Holst JJ. Incretin hormone secretion in women with polycystic ovary syndrome: Roles of obesity, insulin sensitivity, and treatment with metformin. *Metabolism.* 2009;58:586-93.
- III.* Rabøl R, Svendsen PF, Skovbro M, Boushel R, Haugaard SB, Schjerling P, Schrauwen P, Hesselink MK, Nilas L, Madsbad S, Dela F. Reduced skeletal muscle mitochondrial respiration and improved glucose metabolism in nondiabetic obese women during a very low calorie dietary intervention leading to rapid weight loss. *Metabolism.* 2009;58:1145-52.
- IV.* Svendsen PF, Madsbad S, Nilas L. The insulin resistant phenotype of polycystic ovary syndrome. *Fertil Steril.* 2010;94:1052-8.
- V.* Svendsen PF, Madsbad S, Nilas L, Paulsen SK, Pedersen SB. Expression of 11 β -hydroxysteroid dehydrogenase 1 and 2 in subcutaneous adipose tissue of lean and obese women with and without polycystic ovary syndrome. *Int J Obes.* 2009;33:1249-56.
- VI.* Svendsen PF, Jensen FK, Haugaard SB, Nilas L, Holst JJ Madsbad S. The effect of a six week very low caloric diet on insulin sensitivity, beta cell function, insulin clearance, incretin hormone secretion, androgen levels and body composition in obese young women. *Sjcli.* 2012;72:410-9.
- VII.* Rabøl R, Svendsen PF, Skovbro M, Boushel R, Schjerling P, Nilas L, Madsbad S, Dela F. Skeletal Muscle Mitochondrial Function in Polycystic Ovarian Syndrome. *Eur J Endocrinol.* 2011;165(4):631-7.
- VIII.* Svendsen PF, Christiansen M, Hedley PL, Nilas L, Pedersen SB, Madsbad S. Adipose expression of adipocytokines in women with polycystic ovary syndrome. *Fertil Steril.* 2012;98:235-41.

Abbreviations

AIRg , acute insulin response to intravenous glucose administration
AUC, area under the curve
BMI, body mass index
CRP, C-reactive protein
CVD, cardiovascular disease
CT, computed tomography
DEXA, dual energy X-ray absorptiometry
DHEAS, dehydroepiandrosterone sulphate,
DI, disposition index
DPP-4- dipeptidyl peptidase-4
FFA, free fatty acids
GDR, glucose disposal rate
GIP, glucose-dependent insulintropic peptide
GLP-1, glucagon-like peptide-1
GOX-1, basal glucose oxidation
GOX-2, insulin stimulated glucose oxidation
HOMA-IR, homeostatic model assessment insulin resistance index
IGF, insulin-like growth factor
IGF-1, insulin-like growth factor-1
IGFBP, insulin-like growth factor binding protein
IGFBP-1, insulin-like growth factor binding protein-1
IL-6, interleukin-6,
IR, insulin resistance
ISI, insulin sensitivity index
IVGTT, intravenous glucose tolerance test
LH, luteinising hormone
LIPOX-1, basal lipid oxidation
LIPOX-2, insulin stimulated lipid oxidation
NIH, the National Institutes of Health
NOGM, non-oxidative glucose metabolism
OGTT, oral glucose tolerance test
PCOM, polycystic ovarian morphology
SHBG, sex hormone binding globulin
SI, insulin sensitivity
TNF- α , tumour necrosis factor alpha
T/P, trunk/peripheral fat
TG, triglycerides
VLCD, very low caloric diet

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1. Introduction

The polycystic ovary syndrome (PCOS) is a heterogeneous disease of poorly understood aetiology characterised by hyperandrogenemia and chronic anovulation. The manifestations of PCOS vary depending on ethnicity, environmental factors and the presence and severity of obesity and insulin resistance. The most common clinical features of PCOS are menstrual dysfunction, infertility, hirsutism, acne, alopecia, visceral fat distribution and obesity.

The true prevalence of PCOS is unknown, but an estimated 5 to 7% of young fertile women have the disease, which makes it the most common endocrinopathy amongst young fertile women and the most common cause of female infertility.

Although the reproductive consequences of PCOS have been recognised for at least 70 years, there has, more recently, been considerable focus on the metabolic consequences of PCOS. Burghen et al. were the first to report that women with PCOS were hyperinsulinemic, which suggests the presence of insulin resistance (IR) (1). This was subsequently confirmed by several other authors (2-6). Dunaif et al. have shown that women with PCOS have profound peripheral IR independent of obesity (7), which may be caused by a unique, apparently genetic, disorder of insulin action (8-10).

Persistent peripheral IR in combination with beta cell secretory defects leads to glucose intolerance (11). However, not all women with PCOS and peripheral IR develop impaired glucose tolerance because as long as the beta cell can compensate for the insulin resistance, glucose tolerance remains normal.

A family history of type 2 diabetes is a well-known risk factor for glucose intolerance and type 2 diabetes, and there is evidence that beta cell dysfunction is more pronounced in women with PCOS who have a family history of type 2 diabetes (12;13).

In recent years, there has been an increased focus on fetal programming and the intrauterine environment that seems to play a role in the development of IR and PCOS in the offspring. Thus epidemiological observations and animal studies have shown that the abnormal nutritional, hormonal and metabolic environment provided by the mother may program target tissues, i.e. muscle, pancreatic beta cells and fatty tissue in the offspring, towards the development of the metabolic syndrome/PCOS phenotype in adult life (14-17). In line with this, there is increasing evidence indicating that PCOS may have marked implications for pregnancy outcomes and the long-term health of a woman and her offspring (18-21).

Obesity, in particular upper-body obesity, plays a central role in the development of PCOS, but the association between obesity and the PCOS phenotype is not fully understood, and it is still debated whether obesity is a cause or a consequence of PCOS. We hypothesised that different pathophysiological mechanisms account for the development of PCOS in lean versus obese women.

Thus, one of the purposes of our studies was to differentiate between the effect of PCOS and the effect of obesity on relevant metabolic parameters. The studies evolved around IR, which is considered a key factor in the pathophysiology of PCOS and other obesity-related diseases, e.g. type 2 diabetes and cardiovascular disease. We investigated some of the causes and consequences of IR, with particular focus on the adipose tissue as an endocrine organ. We hypothesised that some of the molecular biological processes that take place in fatty tissue may be the link between the distribution of adipose tissue and IR. We consider the investigation of this link to be a very important issue in investigations of PCOS, type 2 diabetes and cardiovascular disease. We have also investigated metabolic processes in muscular tissue, which is the primary site of peripheral IR. Defects in insulin signal transduction have been demonstrated in women with PCOS (7-9). We

looked at the effect of obesity and IR on mitochondrial function, which we hypothesised, is impaired in women with PCOS, as has been shown to be the case in obesity and type 2 diabetes. In order to achieve more knowledge about the development of mitochondrial impairment in obesity, we also studied the effect of weight loss on mitochondrial function in obese subjects without co-morbidities.

Thus, the primary aims of our studies were to investigate:

- The independent effect of PCOS and obesity on different physiological mechanisms of glucose and insulin metabolism.
- Enzymatic processes involved in cortisol metabolism in PCOS and obesity.
- Adipose expression and plasma levels of adipocytokines in PCOS and obesity, and how they are associated with various measures of insulin sensitivity (SI) and body composition.
- Incretin hormones secretion in PCOS and obesity, and the effect of metformin on incretin hormone secretion in lean and obese women with PCOS.
- The effect of weight reduction on secretion of incretin hormones and insulin metabolism and incretin hormone secretion.

- Mitochondrial function in PCOS and obesity.
- Mitochondrial function in obese healthy women before and after weight reduction.

All the studies included in the thesis are based on a population consisting of 21 obese women with PCOS, 19 lean women with PCOS, 16 obese control women and 9 lean control women. The number of study subjects varied slightly between the different studies because of laboratory technical problems or lack of compliance. All PCOS women and the obese control group were recruited by advertising in the local newspaper. The PCOS women were offered participation in the study if they had the following symptoms: oligo-amenorrhoea and/or hirsutism. The majority of women who applied had un-diagnosed PCOS. Women who were already in treatment for PCOS were excluded. We used the Rotterdam criteria for diagnosing PCOS, knowing that this gave us a very heterogenic study population, but which we assumed would be representative of the PCOS population, and well aware that some of results regarding pathophysiological mechanisms may have been diluted by including women with less severe symptoms of PCOS. The study population is described in detail in paper IV. The obese control women were recruited primarily for a weight

loss project, but their baseline data were also used to match the obese PCOS group.

We used gold standard methods for evaluation of the different metabolic parameters. Thus, SI (in peripheral muscle) and beta cell function were evaluated by use of a "Botnia" clamp (22), which consists of an intravenous glucose tolerance test (IVGTT) followed by an euglycaemic hyperinsulinemic clamp. In the fasting state and during the steady state period of the clamp (after 2 hours), we performed muscle and fat biopsies.

To assess SI, we also calculated the homeostasis model assessment insulin sensitivity index (HOMA-IR) from fasting insulin and glucose. The HOMA-IR is primarily an index of hepatic insulin sensitivity. We also investigated glucose (GOX) and fat (LIPOX) oxidation and non-oxidative glucose metabolism, which were performed by indirect calorimetry before and during the euglycemic clamp.

We investigated incretin secretion by use of a frequently sampled 3-hour oral glucose tolerance test (OGTT), which at the same time gave us a measure of glucose tolerance (blood glucose at 0 and 120 min). We measured total GLP-1 and GIP as indexes of incretin hormone secretion.

Mitochondrial function was measured in permeabilized skeletal muscle fibres. This method allowed us to perform detailed measurements of the electron transport chain and study mitochondrial oxygen consumption *ex vivo* using high-resolution respirometry. Cortisol metabolism was evaluated using adipose expression of the enzymes 11 β -HSD-1 and 2 and H6PDH, which are involved in the inter-conversion of cortisone to cortisol. Body fat distribution was evaluated by Dual X-ray absorptiometry (DEXA) scan for the evaluation of whole body fat distribution in combination with a computed tomography (CT) scan for the evaluation of intra-abdominal and subcutaneous fat distribution.

2. The PCOS diagnosis and insulin resistance

2.1 The Rotterdam criteria

There has been much debate about the diagnostic criteria for PCOS. From 1990 to 2003, the National Institutes of Health (NIH) criteria were used (23), which included the following symptoms: clinical or biochemical evidence of hyperandrogenism, chronic anovulation and exclusion of other known disorders. These criteria were based on a general consensus rather than on scientific evidence. In 2003, a consensus conference was held in Rotterdam in order to revise the

existing diagnostic criteria, and the following diagnostic criteria were agreed on (24):

- Oligo- or amenorrhoea
- Clinical and/or biochemical signs of hyperandrogenism
- Polycystic ovaries by transvaginal ultrasonography
 - and exclusion of other aetiologies

The criteria were broadened with polycystic ovarian morphology (PCOM), but the relevance of these added criteria is debated (25-28). Polycystic ovarian morphology is a very common finding amongst "normal" women (29), and the inclusion of PCOM in the diagnostic criteria for PCOS may lead to over-estimation of the prevalence of PCOS, and may lead to dilution of the severity of PCOS-related diseases, because women with e.g. anovulation and PCOM may not have the same metabolic disturbances as women with hyperandrogenemia and anovulation. We addressed this issue by investigating the association between the different physiological traits of glucose/insulin metabolism and the different PCOS phenotypes. We found the strongest association between androgen levels and SI. No associations were found between PCOM, menstrual pattern and the various measures of SI (30). These findings should be kept in

mind when treating and advising women with PCOS and should lead to further investigation, discussion and re-evaluation of the Rotterdam criteria.

2.2. PCOS, insulin resistance, beta cell function and obesity

IR is considered the key pathophysiological defect in the development of the PCOS phenotype. Dunaif and co-workers were the first to report a profound defect in insulin signal transduction in peripheral skeletal muscle in women with PCOS independent of obesity (7). This could explain the hyperinsulinemia that characterises women with PCOS, and possibly also the induction of β -cell dysfunction that has been demonstrated in some women with PCOS. But how is this linked to anovulation and hyperandrogenemia? It has been suggested that the pathophysiological mechanisms may take their origin in the ovary or in the hypothalamic-pituitary axis (31-34). Insulin may stimulate androgen synthesis from the ovarian theca cells through its interaction with the insulin-like growth factor (IGF) system and that IGF-I potentiates luteinising hormone (LH)-stimulated ovarian androgen synthesis. The action of IGF-I is modulated by the IGF-binding protein-1 (IGFBP-1) (35), the level of which is inversely correlated with the plasma level of free IGF-I (36). Insulin has also been

shown to regulate the plasma level of IGFBP-1 (37) by decreasing hepatic production (38). Because plasma IGFBP-1 is decreased in women with PCOS (36;39;40), it has been hypothesised that hyperinsulinemia in PCOS inhibits IGFBP-1 production, leading to increased plasma levels of free IGF-I, potentiating LH-stimulated androgen production.

3. Adipose tissue

3.1. PCOS, obesity and adipocytokines

Sixty to seventy percent of women with PCOS are overweight or obese (41), and obesity plays a major role in the development and severity of PCOS (42-45).

It has long been recognised that nutritional status and body weight can have profound effects on reproductive function. The mechanisms of the adverse influence of under nutrition that leads to disturbances in the hypothalamic control of gonadotrophins by gonadotrophin releasing hormone (GnRH) are reasonably well understood (46).

However, the effects of obesity on reproductive function are more complex, and there is little evidence for a primary disturbance in the hypothalamic pituitary axis. It seems more likely that peripheral effects, e.g. release of substances from the adipose tissue, may have a direct effect on the ovary.

Obesity and insulin resistance are associated with a state of chronic “low-grade” inflammation, manifested by increased levels of molecular mediators of inflammation (CRP, TNF- α and IL-6), (47-49).

Furthermore, these substances have shown to be independent predictors of type 2 diabetes and cardiovascular disease (50-53). Kelly et al. were the first to report low-grade chronic inflammation in women with PCOS based on their finding of increased levels of CRP in women with PCOS compared with controls (54). Since then, several authors have investigated the role of inflammation, but the results are incongruent (55-63). Leptin and adiponectin are adipose-derived hormones that have been associated with obesity, insulin resistance and the metabolic syndrome.

Adiponectin has insulin-sensitising, anti-atherogenic and anti-inflammatory actions (64;65), and lower levels of adiponectin have been demonstrated in obese patients and in patients with type 2 diabetes and coronary artery disease (66-68). In PCOS, the role of adiponectin has been debated. Some authors have found decreased levels of adiponectin in PCOS (51;69-77), whereas others have not (70;78-82). Leptin is involved in the regulation of energy homeostasis (83-85) and has also shown to play a role in reproductive physiology (86). There is abundant evidence that leptin may be an important link between

metabolism and reproduction (86). Increasing plasma levels of leptin have been found with increasing body weight, explained by the development of hypothalamic leptin resistance.

In paper VIII, we investigated the adipose expression of leptin, adiponectin and IL-6 and plasma levels of leptin, adiponectin, visfatin, resistin and TNF- α in lean and obese women with and without PCOS (87). We found no independent effect of PCOS on either the adipose expression of or the circulating levels of the above-mentioned adipokines (87); however, obesity was independently associated with increased adipose expression and plasma levels of leptin and a lower adipose expression of adiponectin (87). The role of adipocytokines in the pathogenesis of PCOS, where obesity is a major confounder, remains controversial, and future investigations addressing this issue should also focus on the lean PCOS phenotype.

3.2. PCOS and central obesity

Women with PCOS are often characterised by upper body obesity (60;88;89), which is now recognised as a major pathogenic factor in the development of IR and obesity-related disease such as type 2 diabetes and cardiovascular disease (CVD) (90-92). Visceral fat in non-obese women with PCOS contributes

markedly to high serum triglyceride (TG) and fasting insulin levels (93;94).

Dysregulation of free fatty acid (FFA) release, especially from upper body subcutaneous adipose tissue, appears to contribute substantially to the metabolic disturbances associated with obesity. Previous studies have shown that elevated plasma levels of FFAs enhance hepatic glucose production and impair muscle glucose uptake, oxidation and storage (95). Free fatty acids also affect insulin secretion (96) and beta-cell function (97;98), and elevated levels of FFA have been associated with ischemic heart disease (99).

It is feasible that the PCOS-associated upper body fat may be linked to hyperandrogenism/hyperandrogenemia, possibly through differences in sex steroid metabolism in visceral and subcutaneous fat. On the other hand, we know that many of the symptoms that characterise PCOS (anovulation and hyperandrogenism/hyperandrogenemia) may be caused by obesity alone, but 30–40% of women with PCOS are lean, indicating that there may be different pathophysiological mechanisms behind the development of PCOS in lean versus obese women.

In order to investigate the independent effect of PCOS and obesity on the hormonal and metabolic dysfunctions in these women, we

investigated various measures of insulin and glucose metabolism in 36 (19 obese and 17 lean) women with PCOS and 25 (16 obese and 9 lean) weight-matched control women. Women with PCOS had lower peripheral and whole body SI compared to weight-matched control women (100). This may partly be explained by the relatively increased upper body obesity associated with PCOS (42;94;101;102). Women with PCOS also had lower insulin stimulated glucose oxidation (GOX-2) and non-oxidative glucose metabolism (NOGM) as assessed by indirect calorimetry. The adaptation of beta cell function to the prevailing SI was assessed by the acute insulin response to an IVGTT and calculation of the disposition index (DI). We calculated the independent effect of PCOS and obesity, and found no independent effect of PCOS on either the acute insulin response to glucose infusion (AIRg) or to the DI, indicating a normal β -cell adaptation to SI. However, increased AIRg and decreased DI were independently associated with obesity. From this study, we conclude that PCOS is associated with several metabolic disturbances independent of obesity, but that obesity is associated with more severe metabolic disturbances than observed in lean women with PCOS (103). However, when lean women with PCOS become obese, some of their metabolic disturbances, e.g. SI, are

more severe than in weight-matched women with obesity without co-morbidities (103). This phenomenon was described as a negative additive synergistic effect of PCOS and obesity. We also investigated fasting and insulin-stimulated suppression of FFA in our population, and we found that they did not differ between the groups with and without PCOS, which may be explained by the relatively young age and relatively short duration of IR in our subjects.

3.3. PCOS and 11 β -hydroxysteroid dehydrogenase types 1 and 2 (11 β -HSD1 and 2)

Glucocorticoids play an important role in the pathogenesis of obesity and IR. Circulating concentrations of cortisol are not elevated in obesity, but it has recently been demonstrated that the conversion of cortisone to cortisol in adipose tissue is increased in obesity (104). Two isoenzymes of 11 β -hydroxysteroid dehydrogenase catalyse the interconversion of the inactive steroid cortisone to the active glucocorticoid cortisol. The enzyme 11 β -hydroxysteroid dehydrogenase-2 (11 β -HSD2) inactivates cortisol to cortisone in the distal nephron, whereas 11 β -hydroxysteroid dehydrogenase-1 (11 β -HSD1) regenerates cortisol from cortisone, primarily in liver and adipose tissue. Eleven β -hydroxysteroid dehydrogenase seems to play an important

role in the development of metabolic disease. Thus increased enzyme activity has been found in adipose tissue in obesity, whereas inhibition of 11 β -hydroxysteroid dehydrogenase protects against development of obesity and enhances insulin sensitivity. Glucocorticoid metabolism in PCOS has been sparsely investigated, but it has been suggested that defective 11 β -HSD1 activity may contribute to its pathogenesis. A recent demonstration of a polymorphism in the HSD11B1 gene, which encodes 11 β -HSD1, has led to the suggestion that a genetic variation in 11 β -HSD1 contributes to enhanced cortisol clearance (105). In contrast to these results, we demonstrated an independent increase in the expression of 11 β -HSD1 in subcutaneous adipose tissue in PCOS and obesity. These findings support the theory of an altered glucocorticoid metabolism in PCOS. Further investigations are needed to reveal whether a proposed dysregulation of glucocorticoid metabolism is a cause or a consequence of PCOS.

4. Muscular tissue

4.1. Insulin signalling-mechanisms of insulin resistance

Insulin regulates numerous post-prandial events, among which, controlling the cellular localisation of the glucose transporter GLUT4 in muscle and fat is a central mechanism in

the management of blood glucose homeostasis. In the basal state, GLUT4 resides in an intracellular membrane compartment, and, after insulin stimulation, it is rapidly transferred to the plasma membrane where it permits cellular glucose influx. In individuals with IR, this process is impaired, leading to inappropriate response of peripheral muscle and fat to physiological levels of insulin. The activation of GLUT4 is preceded by a cascade of events initiated by the binding of insulin to its receptor, composed of two disulphide-linked heterodimers, each of which has an α and β subunit. Insulin binding to its receptor leads to autophosphorylation and activation of tyrosine kinase, resulting in subsequent phosphorylation of several intracellular substrates (106). Insulin receptor substrate-1 (IRS-1) was the first docking protein identified in the IRS family (107). Tyrosine-phosphorylated IRS-1 protein transduces downstream signals by directly binding to the SH2 domains of various signalling proteins, including phosphatidylinositol 3-kinase (PI 3-Kinase). The activation of PI-3 kinase is one of the earliest steps in the insulin-signalling pathway (108) and plays a major role in many insulin-regulated responses, including stimulation of glucose uptake (109), glycogen synthesis (110), antilipolysis (111), protein synthesis (112), and gene expression. Insulin resistant

states such as obesity and type 2 diabetes are associated with defects at different sites in the insulin transduction cascade. Thus type 2 diabetes has been associated with diminished insulin-stimulated IRS-1 tyrosine phosphorylation and decreased PI3K activity coupled to impaired glucose transport (113). Likewise, skeletal muscle and adipocytes from obese, insulin-resistant individuals demonstrate impaired insulin-triggered IRS-1-associated PI3K activity compared to lean individuals (114;115). Women with PCOS have profound IR, which may be explained by a post-binding defect in insulin signalling in adipocytes and skeletal muscle. It has been suggested that this defect may be due to constitutive serine phosphorylation, which inhibits insulin-stimulated tyrosine phosphorylation (116).

4.2. PCOS, obesity and mitochondrial function

Obesity is associated with anomalies in mitochondrial energy production and impaired cellular energy metabolism, implying diminished fat oxidation that results in increased ectopic storage of TGs and a greater dependence on glucose for ATP synthesis in skeletal muscles. This is reflected in the finding of higher intra-myocellular lipid content and a lower rate of mitochondrial ATP production in muscle in insulin-resistant

subjects compared to control subjects with normal SI (117). Thus, reduced skeletal muscle mitochondrial lipid oxidation may be the link between obesity and the comorbidities associated with it. It is, however, controversial whether intra-myocellular lipid accumulation is a cause or a consequence of decreased mitochondrial function (118). Mitochondrial function has not previously been investigated in women with PCOS, but because the metabolic disturbances in women with PCOS resemble those of obesity and type 2 diabetes, we hypothesised that mitochondrial function would be impaired in these women. Thus, we tested the hypothesis that skeletal muscle mitochondrial respiratory capacity is reduced in PCOS, linking mitochondrial impairment to the muscular insulin resistance that is a central pathophysiological trait of PCOS. We applied the method of high resolution respirometry (119) on permeabilized skeletal muscle fibres of women with PCOS in order to thoroughly characterise mitochondrial respiration. High resolution respirometry allows detailed measurements of electron transport chain function through successive addition of substrates and inhibitors. As described in the next section, we tested mitochondrial function before and after weight loss.

4.3. Obesity and mitochondrial function—effect of weight loss

The increasing prevalence of obesity has resulted in an increased interest in diet-induced weight loss, which remains the cornerstone of the treatment of obesity. A very low caloric diet (VLCD) is a safe way of losing weight and can be used as a method of investigating the impact of body weight on metabolic risk markers and pathophysiological mechanisms.

We gave an 8-week, very-low-caloric diet (500-600 kcal/day provided by NUPO formula) to obese healthy women with an average BMI of 33.4 kg/m². After the 8-week intervention period, the women had lost an average 11% of their initial body weight, with a significant reduction in subcutaneous abdominal adipose tissue and a borderline significant reduction in visceral abdominal adipose tissue. Peripheral SI was unaltered, but there was a significant improvement in HOMA-IR, which is primarily an index of hepatic SI. We also evaluated androgen levels before and after weight loss and found a significant decrease in free testosterone, which was explained by a significant increase in SHBG. We investigated mitochondrial respiration before and after the weight loss and found decreased mitochondrial function, whereas the intra-myocellular TG content was unchanged, in line with the unaltered

peripheral insulin. We investigated mitochondrial respiration before and after weight loss and found decreased mitochondrial function, whereas the intra-myocellular TG content was unchanged, in line with the unaltered peripheral SI. We investigated mitochondrial respiration before and after the weight loss and found decreased mitochondrial function after weight loss, whereas the intra-myocellular TG content was unchanged, in line with the unaltered peripheral insulin.

Weight reduction and the concomitant improved insulin sensitivity are expected to increase mitochondrial capacity, in line with the observations of Kern and co-workers (120) and Toledo and co-workers (121;122). However in a recent study, the latter were unable to demonstrate any effect of weight loss alone on mitochondrial content and electron transport chain activity (123). The decrease in mitochondrial function after drastic weight loss may be explained by the fact that our subjects were studied while they were still dieting and therefore still in a catabolic state. Because of this, the intermediate metabolism and energy expenditure of these subjects may have been different from that in people who are in metabolic steady state. The regulation of mitochondrial activity is still poorly understood, and more investigations are

needed to clarify the relationship between mitochondrial regulation and obesity-related disease.

5. Beta cell function

5.1. Beta cell function and incretin hormones

When glucose is administered orally, insulin secretion is stimulated much more than it is during an isoglycemic intravenous glucose load. This effect, which is called "*the incretin effect*", is estimated to be responsible for 50–70% of the insulin response to oral glucose, and is caused mainly by the two intestinal insulin-stimulating hormones: glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide-1 (GLP-1). In patients with type 2 diabetes, the incretin effect is either greatly impaired or absent (124), and it is assumed that this contributes to impaired beta cell function in people with glucose intolerance. In type 2 diabetic patients, a normal secretion of GIP has been found, whereas the secretion of GLP-1 is reduced in some but not all studies (124). However, the effect of GIP on insulin secretion is severely impaired, whereas the effect of GLP-1 is partly preserved. Several other conditions involving IR and glucose intolerance have been associated with altered incretin effect (124-128), but there has been some debate about the effect of obesity on the incretin effect. In a recent study, Muscelli et

al. found that obesity and glucose tolerance independently attenuate the GLP-1 response during an oral glucose tolerance test.

5.2. PCOS, metformin and incretins

Incretin hormone secretion has been sparsely investigated in women with PCOS, but since the pathophysiological mechanisms of the disease resemble those of type 2 diabetes, it is reasonable to assume that alterations in incretin secretion or action may be found in women with PCOS. We were, however, not able to demonstrate any differences in incretin hormone secretion between women with and without PCOS. To our knowledge, only one previous study has evaluated incretin hormone secretion in women with PCOS. Vribikova et al. found significantly higher levels of total GIP in women with PCOS compared to control women during an OGTT, but lower levels of GLP-1 during the late phase of the OGTT in women with PCOS (129). These investigators used a slightly different methodology than we did, which may explain the different results. Metformin belongs to the biguanide class of oral antidiabetic agents and is widely used in the treatment of both type 2 diabetes and PCOS. In PCOS, metformin reduces IR, improves ovarian function, regulates cycles, lowers androgens, improves clinical hyperandrogenism and potentially improves

fertility. Metformin is also likely to delay diabetes onset and has a role in PCOS in those individuals at high risk of diabetes.

Furthermore, several studies have suggested that metformin may actually increase GLP-1 biosynthesis and secretion from the intestinal L-cells and decrease dipeptidyl peptidase IV (DPP-IV) (130-133) activity, resulting in a higher plasma concentrations of active GLP-1 (133;134). Dipeptidyl peptidase IV (DPP-IV) is a serine protease that cleaves the penultimate proline or alanine at the N-terminus of several polypeptides, amongst others glucagon-like peptide-1 (GLP-1 and GIP). Rapid inactivation of GLP-1 and GIP by DPP-IV limits its ability to enhance glucose-induced insulin secretion and improve glucose tolerance.

We investigated the effect of metformin on incretin secretion in lean and obese women with PCOS, and we found a significant increase in both GIP and GLP-1 in lean women, but unaltered incretin levels in obese women with PCOS after metformin intervention (135).

5.3. Incretins and weight loss in obesity

Due to their physiological effect on energy balance and appetite regulation, the gastrointestinal hormones have attracted increasing interest as obesity drug targets, and there is currently focus on the role of incretin

hormones in the remission of type 2 diabetes after bariatric surgery (136). We evaluated incretin secretion during an OGTT before and after a weight reduction of ~ 11% induced by an 8-week, very low caloric diet, and we found unaltered GLP-1 secretion and increased GIP secretion. Our study population was characterised by an initial average BMI of ~ 33 kg/m² and no concomitant diseases. Although bariatric surgery has the best long-term weight loss results in the treatment of obesity as compared with lifestyle intervention (137;138), the operation is associated with complications such as malabsorption of vitamins and anaemia, and it remains reserved for the treatment of morbid obesity. Furthermore, the long-lasting effects and complications of bariatric surgery remain unknown. Thus, lifestyle intervention remains the cornerstone of the treatment of simple obesity.

6. Long-term consequences of obesity and PCOS

In 1956, the first reports of obesity-related adverse health consequences appeared, and it was noted that they occurred predominantly in those with upper-body fat distribution (139;140). Since then, several investigators have shown that upper-body obesity, in particular visceral obesity, is associated with a cluster of atherogenic metabolic abnormalities

that are now often referred to as the metabolic syndrome (141).

Insulin resistance is now recognised as a major risk factor for the development of type 2 diabetes (142), and it is one of the components of the metabolic syndrome, characterised by central obesity, lipid disturbances, insulin resistance, glucose intolerance and hypertension. The metabolic syndrome identifies patients at increased risk of type 2 diabetes and cardiovascular disease. The prevalence of the metabolic syndrome in women with PCOS under the age of 20 has been reported to be as high as 23% (143), and it is not surprising that the prevalence increases with advancing age. In this same study that the prevalence increased to 53% in women aged 30–39 years. The prevalence of the metabolic syndrome in women with PCOS under the age of 20 has been reported to be as high as 23% (143). And it is not surprising that the prevalence increases with advancing age, prevalence increasing to 53% in women aged 30–39 years in this same study (143).

Pancreatic β -cell dysfunction is a second important risk factor for the development of type 2 diabetes, and impaired β -cell function has also been shown in women with PCOS (13;143;144). Women with PCOS are therefore considered at increased risk of

developing type 2 diabetes, although only a few prospective studies have been able to confirm this (145-149) .

7. Perspectives

The prevalence of obesity has increased dramatically during the last 2 to 3 decades. Globally, there are more than 1 billion overweight adults, of which at least 300 million are obese. The primary causes of obesity are increased consumption of energy-dense foods high in sugars and saturated fats and reduced physical activity. Thus, obesity was initially considered a symptom of an abundance of and easy access to food in the Westernised world, but the epidemic is now spreading to the third world countries as well, which now also have to deal with the obesity-related health consequences.

Obesity and obesity-related morbidity have become a major challenge in the health care system worldwide, and the challenge has become even larger over the last decade, during which the obesity epidemic has also spread to children.

Thus, in Denmark, the prevalence of childhood obesity has increased over 20 fold during the past 5 decades, and one child out of five is overweight (150). This may seem alarming, but in comparison with the United States where currently 9 million school

children are obese (151), these numbers seem negligible.

Childhood obesity is associated with a variety of diseases, such as diabetes, cancer and cardiovascular disease later in life.

Furthermore, childhood obesity is associated with hormonal disturbances leading to pubertas praecox, which again leads to the development of PCOS in females and may also be linked to male infertility. Both male and female infertility are an increasing problem in the Westernised world, and the need for assisted reproduction is increasingly adding an extra burden to the health care system. A large proportion of the infertility problems are caused by obesity-related hormonal and metabolic disturbances, and although obese women may be able to conceive through assisted reproduction, there are several complications (maternal and fetal) associated with obesity in pregnancy, such as gestational diabetes, pre-eclampsia and adverse fetal outcome, that increase the need for prenatal care. This is another incentive to put more action into the area of population-based weight reduction intervention.

Since the first report of PCOS appeared in the 1930s, the prevalence of the disease has increased, not only because of the increased interest in and diagnosis of the disease, but also because of the dramatic increase in

obesity which is so closely associated with PCOS.

Research done during the past decades has shown that PCOS is not an isolated gynaecological disease as initially assumed, but a multi organ disease that involves several medical specialties. Although much research has been carried out in order to investigate the pathogenesis of PCOS, it is still unknown why only a minor proportion of obese and insulin-resistant women develop PCOS.

In 1935, PCOS was a disease of the ovary; in the 1970s, it was a disease caused by abnormalities of the hypothalamic-pituitary-gonadal axis. In the 1980s, the association with insulin resistance was discovered, and this led to an increased focus on pancreatic beta cell function and the risk of developing impaired glucose tolerance and manifest type 2 diabetes. In the early 1990s, a defect in the post-receptor insulin signal transduction was found in women with PCOS, and it was suggested that this was the central pathophysiological mechanism of the disease. However, later in the 1990s, it was demonstrated that fatty tissue and body fat distribution also play a role in the PCOS pathophysiology.

There seems to be different pathophysiological mechanisms involved in the different phenotypical expressions of the disease. Thus, women with PCO morphology

and anovulation but androgen levels within the normal range may not have the same pathophysiology as women with hyperandrogenemia and anovulation.

Furthermore, obese and lean women with PCOS also seem to have developed the disease on the basis of different pathophysiological mechanisms.

Nevertheless, PCOS may be a consequence of obesity, and the symptoms may improve after weight reduction. On the other hand, not all obese women develop PCOS, and we have shown that several metabolic and hormonal dysfunctions are associated with PCOS, independent of obesity. We have also shown that the presence of both PCOS and obesity has a negative synergistic effect on several hormonal and metabolic parameters. We have discussed the current diagnostic criteria for PCOS and evaluated their suitability for identifying the women with PCOS who are at increased risk of developing long-term comorbidity and mortality due to PCOS. We demonstrated that androgen levels were the strongest predictor of insulin sensitivity, and we found no associations between anovulation, PCOM and the various measures of SI. On this background, we conclude that the current diagnostic criteria for PCOS include women who may not be at increased risk of developing long-term consequences of the disease in terms of type 2 diabetes and

cardiovascular disease. There are other long-term risks of anovulation, such as endometrial cancer and osteoporosis, which means that in daily clinical practice, the different phenotypical presentations of PCOS should be addressed and informed about the long-term consequences in accordance with their clinical presentation of PCOS.

The long-term consequences of PCOS have been sparsely investigated, with only a very few prospective studies addressing this issue. Women with PCOS have all the metabolic risk factors associated with long-term increased morbidity and mortality, and PCOS should be considered a serious metabolic disease.

At present, we are focusing on treating the symptoms and risk factors of PCOS. Oral contraceptives regulate the bleeding pattern and decrease androgen levels, but may, on the other hand, have a pejorative effect on glucose/insulin/lipid metabolism. They are, however, widely used in the treatment of PCOS. In 1994 metformin was introduced in the treatment of PCOS, and it was shown to induce ovulation and improve objective measures of hirsutism. The use has been extended to pregnant women with PCOS, in whom metformin may prevent early miscarriage and gestational diabetes.

It remains, however, unknown how all the effects of metformin are mediated in PCOS. We know that metformin improves insulin sensitivity especially in the liver, but metformin also seems to have an effect in lean women with PCOS with normal insulin sensitivity. The glitazones have a pronounced effect on insulin sensitivity and have also been shown to ameliorate the signs and symptoms of PCOS, underscoring the importance of insulin resistance in the pathogenesis of PCOS.

Several studies have already investigated the effect of lifestyle and pharmacological intervention; all of which have at best moderate and short-term effects on the key defects of PCOS. Other areas of research could be directed at environmental factors, e.g. chemical and hormonal agents that may predispose to the development of PCOS. Focus could be directed towards epigenetic mechanisms in the pathogenesis of PCOS, focusing on the intrauterine environment and fetal exposure to agents that may disturb the epigenetic programming in fetal reproductive tissue and thereby cause development of PCOS later in life. Thus, animal studies have shown that intrauterine exposure to androgens is associated with development of a PCOS-like phenotype (17;152).

Lastly, we suggest a major revision of the Rotterdam criteria in order to reduce the number of phenotypical presentations of PCOS, so that the focus can be on those women with an increased risk of long term morbidity.

8. Summary and comments

Polycystic ovary syndrome (PCOS) is the most common endocrine disorder in young women and the most common cause of female infertility. There is a close link between obesity, insulin resistance and the development of PCOS, but the pathophysiological mechanisms behind the development of this disease are only sparsely understood. This thesis is based on a cross-sectional study and two interventions (dietary and metformin) carried out in a cohort of lean and obese women with and without PCOS. The purpose was to investigate the independent effect of PCOS and obesity on some of the pathophysiological mechanisms behind the development of PCOS and obesity. With the use of gold standard methods, we have investigated peripheral insulin sensitivity, beta cell function, oxidative and non-oxidative glucose metabolism. We have investigated incretin secretion both before and after metformin intervention and after dietary intervention in obese women without PCOS. There has been particular focus on some of the metabolic processes taking place in adipose tissue that play a role in the pathophysiology of insulin resistance. We have investigated adipose expression of adipocytokines and 11 β -hydroxysteroid dehydrogenase types 1 and 2 (11 β -HSD1 and 2) - two enzymes involved in cortisol

metabolism. Finally, we have investigated mitochondrial function in muscular tissue. Overall, we conclude that PCOS is associated with metabolic disturbances in several organs, although these disturbances seem to be more severe in obese women without PCOS than in lean women with PCOS. But the presence of both PCOS and obesity seems to have a synergistic effect on the metabolic disturbances in glucose, insulin and lipid metabolism. In regard to cortisol metabolism, we demonstrated increased expression of 11 β -HSD1 in subcutaneous adipose tissue in PCOS, independent of obesity and in obesity independent of PCOS. These findings support the theory of an altered glucocorticoid metabolism in PCOS that may contribute to metabolic disturbances in glucose-insulin metabolism.

We have showed that a very low caloric diet (VLCD) is an effective weight loss method that, not only has dramatic effect on the metabolic disturbances associated with obesity, but also results in a decrease in androgen levels. Before commencing these studies, we hypothesised that PCOS was associated with more severe metabolic disturbances than we have been able to demonstrate. However, we chose to use the Rotterdam criteria uncritically, and as we have noted, these include several PCOS phenotypes. This may have had a “delusional”

effect on our results. Therefore, we suggest a revision of the Rotterdam criteria.

9. Danish Summary

Denne afhandling er baseret på en tværsnitsundersøgelse og to interventiosstudier (metformin og diæt), der havde til formål at belyse forskellige patofysiologiske mekanismer bag udviklingen af fedme og polycystisk ovarie syndrom (PCOS). Hensigten var at undersøge den uafhængige effekt af hhv. fedme og PCOS på forskellige patofysiologiske mekanismer med overordnet fokus på insulin resistens, som er central i udviklingen af PCOS. Ved hjælp af guld standard metoder har vi undersøgt perifer insulinfølsomhed, betacellefunktion, oxidativ, og non-oxidativ glukosemetabolisme. Vi har undersøgt inkretinsekretion ved PCOS før og efter metforminbehandling. Vi har fokuseret på nogle af de patofysiologiske mekanismer, der finder sted i fedtvæv, og som er af betydning ved udvikling af insulin resistens. I denne forbindelse har vi undersøgt adipøs ekspression af 11 β -hydroxysteroid dehydrogenase type 1 and 2 (11 β -HSD1 and 2)-to enzymer, der spiller en central rolle i cortisolmetabolismen. Endelig har vi undersøgt mitokondriefunktion ved PCOS samt efter diæt. Overordnet kan vi

konkludere, at PCOS er associeret med metaboliske forstyrrelser lokaliseret til flere organer, selvom disse forstyrrelser ser ud til at være mere alvorlige hos adipøse raske kvinder end hos slanke kvinder med PCOS. Kombinationen af både adipositas og PCOS ser derimod ud til at have negativ synergistisk effekt på de metaboliske forstyrrelser i glukose-, insulin-, og lipidmetabolismen. I forhold til cortisolmetabolismen fandt vi øget ekspression af 11 β -HSD1 i subkutant fedtvæv hos kvinder med PCOS uafhængigt af obesitas og ved obesitas uafhængigt af PCOS. Disse fund støtter teorien om ændret cortisolmetabolisme ved PCOS, der kan være medvirkende til de metaboliske forstyrrelser i glukose-, insulin-, og lipidmetabolisme. Vi har vist at en very low caloric diet (VLCD) er en effektiv vægttabsmetode, der ikke blot har eklatant effekt på de metaboliske forstyrrelser forårsaget af adipositas, den fører også til et fald i androgenniveauet. Initielt var vores teori, at PCOS var associeret med større metaboliske forstyrrelser, end vi har kunnet demonstrere. Vi valgt at anvende de gældende diagnostiske kriterier for PCOS, Rotterdam-kriterierne, og har ukritisk inkluderet alle, der opfyldte disse kriterier. Ifølge ét af vores studier (153) kan dette have haft ”fortyndende” effekt” på resultaterne, og vi opfordrer derfor til revision af disse kriterier.

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