Reproductive and Metabolic Consequences of the Polycystic Ovarian Syndrome

MIRIAM HUDECOVA
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Abstract

Polycystic ovary syndrome (PCOS) is a complex clinical condition characterized by hyperandrogenism and chronic oligo/anovulation. Infrequent ovulation and metabolic alterations in women with PCOS are associated with subfertility and probably increased miscarriage rates compared with normal fertile women. The overall risk of developing type 2 diabetes and impaired glucose tolerance (IGT) is three- to sevenfold higher in PCOS women, and the onset of glucose intolerance seems to occur at an earlier age than in healthy controls. Women with PCOS also have several risk factors for cardiovascular disease, although it is unclear whether they actually experience more cardiovascular events than other women. Very few studies assessing the long-term reproductive and metabolic consequences in older women with previously confirmed PCOS have been conducted. In this long-term follow-up of women with PCOS, 84 women with a diagnosis of PCOS between 1987 and 1995 and age at the follow-up > 35 years and an age-matched population-based group of control women participated. Data on reproductive outcome, ovarian reserve, endothelial function, insulin sensitivity and beta-cell function were collected. According to our results most women with PCOS had given birth and the rate of spontaneous pregnancies was relatively high. The rate of miscarriages was not increased in PCOS patients and the ultrasound findings together with increased levels of anti-müllerian hormone suggested that their ovarian reserve is superior to women of similar age. PCOS women displayed signs of endothelial dysfunction, but this was largely due to the increased prevalence of independent risk factors for cardiovascular disease such as increased BMI, triglycerides and blood pressures. IGT and type 2 diabetes occurred more often in PCOS women. Free androgen levels and beta-cell function decreased over time whereas insulin sensitivity remained unchanged. Obesity at young age and progressive weight-gain rendered them more prone to be insulin resistant at the follow-up. Beta-cell function was increased in PCOS women in comparison with control subjects but declined over time. Independent of PCOS phenotype at the index assessment and persistence of PCOS symptoms at the follow-up investigation, premenopausal women with PCOS had lower insulin sensitivity and increased beta cell function in comparison with control subjects. Conclusion: The long-term reproductive outcomes of PCOS are similar compared to women with normal ovaries. Although symptoms and androgen levels are normalized over time, women with PCOS continue to display reduced insulin sensitivity and increased beta-cell function and they also have an increased risk of IGT and type 2 diabetes.

Keywords: polycystic ovarian syndrome, long-term follow-up, ovarian reserve, anti-Müllerian hormone, insulin sensitivity, early insuline response, impaired glucose tolerance, diabetes, endothelial function, endothelial-dependent vasodilation

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To my family
List of Papers

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


IV Hudecova M, Holte J, Moby L, Olovsson M, Larsson A, Berne C, Sundström-Poromaa I. Prevalence of diabetes, impaired glucose tolerance and insulin sensitivity in women with polycystic ovary syndrome – a long-term follow-up study, manuscript.

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Abbreviations

ACTH  Adrenocorticotropic hormone
AES  The Androgen Excess Society
AI  Augmentation index
AHS  American Heart Association
AMH  Anti-müllerian hormone
ApoA-I  Apolipoprotein A-I
ApoB  Apolipoprotein B
ASRM  American Society for Reproductive Medicine
AUC  Area under the curve
BMI  Body mass index
CHD  Coronary heart disease
hs-CRP  High-sensitivity C-reactive protein
CV  Coefficient of variation
CVD  Cardiovascular disease
DHEAS  Dehydroepiandrosterone
EDV  Endothelium-dependent vasodilation
ESHRE  European Society for Human Reproduction
FAI  Free androgen index
FFA  Free fatty acids
FSH  Follicle-stimulating hormone
GnRH  Gonadotropin-releasing hormone
HDL  High density lipoprotein cholesterol
HOMA-IR  Homeostatic model assessment of insulin resistance
ICSI  Intracytoplasmatic sperm injection
IGFBP  Insulin-like growth factor-binding protein
IGT  Impaired glucose tolerance
IR  Insulin resistance
IVF  In vitro fertilization
IVGTT  Intravenous glucose tolerance test
<table>
<thead>
<tr>
<th>Abbreviation</th>
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<tr>
<td>LDL</td>
<td>Low-density lipoprotein cholesterol</td>
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<td>LH</td>
<td>Luteinizing hormone</td>
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<td>MetS</td>
<td>Metabolic syndrome</td>
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<td>M/I</td>
<td>Insulin sensitivity index</td>
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<tr>
<td>NCEP/ATPIII</td>
<td>The US National Cholesterol Education Program Adult Treatment Panel III</td>
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<tr>
<td>NIH</td>
<td>National Institutes of Health</td>
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<tr>
<td>OGTT</td>
<td>Oral glucose tolerance test</td>
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<td>PCO</td>
<td>Polycystic ovary</td>
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<td>PCOS</td>
<td>Polycystic ovary syndrome</td>
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<td>QUICKI</td>
<td>Quantitative insulin sensitivity check index</td>
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<td>SHBG</td>
<td>Sex-hormone binding globulin</td>
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<tr>
<td>TSH</td>
<td>Thyroid-stimulating hormone</td>
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Introduction

Definition of PCOS

Polycystic ovary syndrome (PCOS) is a complex and heterogeneous clinical condition characterized by hyperandrogenism and chronic oligo/anovulation. It is estimated that approximately 5 - 10% of fertile women have PCOS, although no precise determinations have been possible to obtain [1-5].

The National Institutes of Health (NIH) defined PCOS in 1990 as chronic anovulation or oligomenorrhea, and clinical or biochemical hyperandrogenism [6]. In 2003 revised diagnostic criteria were proposed by the European Society for Human Reproduction and Embryology and the American Society for Reproductive Medicine (ASRM/ESHRE) which also included the typical ultrasonographic appearance of the ovaries [7]. In concordance with the revised diagnostic criteria two out of the three following criteria should be fulfilled for establishing the PCOS diagnosis:

1. Oligo- and/or anovulation
2. Clinical and/or biochemical signs of hyperandrogenism
3. Polycystic ovaries

and exclusion of other etiologies (congenital adrenal hyperplasia, androgen-secreting tumors, Cushing’s syndrome). ASRM/ESHRE consensus defines polycystic ovaries if at least one of the ovaries has at least one of the following: either 12 or more follicles measuring 2-9 mm in diameter, or increased ovarian volume (> 10 cm³). If there is a follicle > 10 mm in diameter, the scan should be repeated at a time of ovarian quiescence in order to calculate volume and area. Thus, in comparison with the NIH criteria, PCOS patients could present with at least one more possible phenotype i.e. with oligo/anovulation and polycystic ovaries (PCO) only.

This led to the initiative of The Androgen Excess Society (AES) and in 2006, the, at the moment last version of PCOS definition was proposed. The AES definition is based on a presence of hyperandrogenism and ovarian dysfunction (expressed as oligomenorrhea/amenorrhea or polycystic ovaries), and exclusion of other androgen excess or related disorders. All possible phenotypes are based on the presence or absence of oligoanovulation, hyperandrogenemia, hirsutism and polycystic ovaries at ultrasound [8], but this definition excludes the previously mentioned
phenotype without hyperandrogenism. In this thesis PCOS is defined according to the Rotterdam criteria, as these criteria were the ones in use when our patients were diagnosed. Because of the ongoing debate on how to best define PCOS, results in this thesis are often also given for subjects fulfilling NIH criteria.

Clinical features of PCOS

Oligomenorrhea and amenorrhea, as signs of anovulation, and hirsutism, as a sign of hyperandrogenism, are the most common complaints of patients with PCOS.

Menstrual cycles longer than 35 days or less then eight menstruation periods per year, are usually applied as definitions of oligo-/amenorrhea [9].

The Ferriman-Gallwey scoring system has been designed to assess the severity of hirsutism. The masculine pattern of body hair growth is described in four degrees on 11 different body places; the upper lip, chin, chest, upper back, lower back, upper abdomen, lower abdomen, arm, forearm, thigh, and lower leg [10]. Although often used in scientific reports on PCOS, the Ferriman-Gallwey score is hampered by the large inter-rater variability and also by the fact that many women have treated themselves prior to clinical evaluation.

The calculation of the free androgen index (FAI) from testosterone and sex steroid hormone binding globulin (SHBG) is widely used for assessment of biochemical hyperandrogenism. As about 80 % of testosterone is bound to SHBG and 19 % to albumin, and only 1 % of testosterone is free and biologically available, total testosterone is not sufficient for evaluation of androgens in women [11].

Pathophysiology of PCOS

The pathogenesis of PCOS is multifactorial. Insulin resistance, defined as a diminished effect of a given dose of insulin on glucose homeostasis, is a highly prevalent feature of women with PCOS. Insulin resistance in PCOS is closely associated with an increase in truncal-abdominal fat mass, elevated free fatty acid (FFA) levels, increased androgens, particularly free testosterone through reduced SHBG levels, and anovulation. The causes of insulin resistance in PCOS are still unknown [12].
There is evidence for a **genetic component** in PCOS based on familial aggregation of cases [13]. Data suggest that PCOS develops as the consequence of an interaction of key genes of ovarian androgen production, with environmental factors, particularly nutritional, or other factors causing hyperinsulinemia [13, 14]. Genes involved in the biosynthesis and metabolism of androgens and those involved in the secretion and action of insulin are, logically, in the centre of interest [15].

Chronic **androgen excess** of ovarian and/or adrenal origin results in **abdominal adiposity** in affected women [16]. Long-term administration of testosterone in female-to-male transsexuals induces abdominal adiposity and insulin resistance [17], and androgen excess during fetal life and infancy is associated with increased risk of developing abdominal adiposity later in life [18-20]. Testosterone applied in supraphysiological doses in women is also followed by insulin resistance [21].

Conversely, abdominal fat and free fatty acids are strongly associated with **insulin resistance (IR)**. The increased flux of FFA from highly lipolytic abdominal fat to the liver and muscles may represent an important link between abdominal obesity and insulin resistance, as elevations of plasma FFA levels inhibit insulin-stimulated glucose utilization [22-24]. Abnormalities in insulin secretion and action might play a role in development and maintenance of adiposity. An overactive early insulin
secretion could stimulate carbohydrate intake and constitute a basis for weight gain [23]. Indeed, overweight (body mass index (BMI) > 25 kg/m²) and obesity (BMI > 30 kg/m²) are very common in this group of women. Between 38 and 88% of women with PCOS are overweight [25], and 10-38% are obese [1, 3, 26]. Insulin resistance induces hyperinsulinemia and subsequently stimulates the ovarian and adrenal hormonal production, inhibits SHBG production, and increases testosterone activity. Gambineri and colleagues have summarized links between hyperinsulinemia and hyperandrogenism in PCOS women as follows:

- Insulin causes a direct stimulation of ovarian androgen secretion, possibly through stimulatory effects on the 17α-hydroxylase/17-20 lyase and on 3β-hydroxysteroid dehydrogenase enzymes
- Insulin decreases levels of SHBG, with concomitant elevation of free androgen tissue availability
- Insulin decreases insulin-like growth factor-binding protein (IGFBP) 1 production, both in the liver and in the ovary
- Insulin upregulates ovarian insulin-like growth factor 1 (IGF-1) receptor type I with amplification of IGF-1, IGF-2 and insulin actions in the ovary
- Insulin increases the luteinizing hormone (LH) ovary receptor number and sensitzes LH secreting pituitary cells to gonadotropin-releasing hormone (GnRH) stimulation
- Insulin promotes ovarian growth and cyst formations
- Insulin can cause a direct stimulation of adrenal androgen secretion, possibly through stimulatory effects on the 17α-hydroxylase/17-20 lyase enzyme
- Insulin may increase the 17α-hydroxylase/17-20 lyase adrenal enzyme response to adrenocorticotropin hormone (ACTH) [27].

Anovulation and polycystic ovaries are the result of disturbances of folliculogenesis. The total number of follicles is determined early in life. Normal follicle development comprises initial recruitment, by which primordial follicles start to mature, and cyclic recruitment, which leads to the growth of a cohort of small antral follicles from which the dominant follicle, destined to ovulate, is subsequently selected [28]. Anti-müllerian hormone (AMH) is expressed in granulosa cells of primary follicles already in the 36th week of pregnancy [29]. AMH continues to be expressed in the growing follicles in the ovary until they have reached the size and differentiation state at which they are to be selected for dominance by the action of pituitary follicle-stimulating hormone (FSH) [30]. The selective
rise in FSH levels that occurs during the luteal to follicular transition appears as a potent stimulus for follicle selection. AMH may prevent multiple selections in the follicle cohort [31]. Granulosa cells start to produce inhibins and estradiol that cause a small but significant and progressive decline in the circulating FSH concentration, due to their inhibitory effects on pituitary secretion [32, 33]. The dominant follicle alone continues to grow. In the absence of pregnancy, luteolysis occurs, the negative feedback of inhibin A and estradiol decreases and the inter-cycle elevation of FSH starts again [31].

The number of primary growing follicles in woman with PCOS and anovulation is six-fold higher than in normal ovulatory women [34]. Serum AMH levels are two to three-fold higher and correlate well with number of antral follicles, assessed by ultrasound [35-37]. This may contribute to the failed dominant follicle selection in women with PCOS [38]. Obesity, insulin resistance, elevated androgens and AMH levels are probably related to the development of the large pool of antral follicles and ovarian volume in women with PCOS. Obesity and insulin resistance may enhance the follicular excess through the dysregulation of AMH or through the pathway of hyperandrogenemia [39].

Reproductive consequences of PCOS

Decreased rates of ovulation and metabolic alterations in women with PCOS are associated with subfertility [40]. Miscarriage rates among women with PCOS are believed to be increased compared with normal fertile women, although supporting evidence is limited. Endometrial abnormalities, poor quality of oocytes, obesity and other intrinsic factors have been suggested to affect implantation and increase the risk for miscarriage [41-43]. However, most studies on miscarriage rates in women with PCOS have been conducted in populations who have undergone assisted reproductive treatment, and the miscarriage rate in unselected PCOS populations is largely unknown.

Insulin resistance, obesity and hyperandrogenism, common in PCOS women, are associated with increased risk of preeclampsia, gestational diabetes, preterm birth and stillbirth [42-44].

Obese women with PCOS, conceiving after in vitro fertilization (IVF) and intracytoplasmatic sperm injection (ICSI), have higher gonadotropin requirements [45], fewer oocytes, a higher miscarriage rate and lower live-birth rate than their non-obese counterparts [46]. Furthermore, neonates coming from these pregnancies have a significantly higher risk of admission to a neonatal intensive care unit and a higher perinatal mortality [47].
Ovarian reserve

The ovarian reserve is the capacity of the ovary to produce fertilizable eggs. Measurements of factors thought to reflect ovarian reserve are used as an important means to predict outcome of assisted reproduction techniques. Variables used to estimate the ovarian reserve include basal or stimulated levels of FSH, AMH, inhibin B and the number of antral follicles and ovarian volume, assessed by means of transvaginal ultrasound [48]. Recently, the menstrual cycle length was also suggested to be independently associated with success rates in assisted reproduction. The chance of delivery after IVF/ICSI was almost doubled for women with a menstrual cycle length of more than 34 days compared with women with a menstrual cycle length less than 26 days [49].

A number of previous reports in young women with PCOS lend support for an increased ovarian reserve in these women compared with age-matched controls [50]. Recently, it was proposed that serum FSH assessments are inferior to measuring AMH in identifying women with reduced ovarian reserve, largely because AMH is menstrual cycle-independent [51]. The ovarian reserve of premenopausal women with PCOS has previously not been established.

AMH

AMH is a dimeric glycoprotein, produced by granulosa cells from pre-antral and antral follicles, which plays an important role in both ovarian primordial follicle recruitment and dominant follicle selection [52]. AMH levels essentially reflect the ovarian follicular pool, as AMH levels gradually decrease throughout reproductive life, are undetectable after the menopause [53, 54], and are unchanged throughout the menstrual cycle [55].

Several studies have reported higher levels of AMH in women with PCOS than in controls [35, 56, 57].

FSH

Results for basal levels of FSH in PCOS have been less conclusive [58], which presumably is the result of the high variability of this hormone across the menstrual cycle and the necessity to restrict the comparisons to strictly cycle-matched samples [59]. Roberts and colleagues suggested that a history of increased basal levels of FSH and normal FSH levels in the beginning of IVF treatment, in women younger than 40 years, predicted lower oocyte yield, but did not significantly compromise the embryo quality. However, in women older than 40 years with prior elevation of basal FSH, both the ovarian response and embryo quality were compromised. Thus, high age in combination with elevated FSH may predict particularly poor IVF outcome.
Interestingly though, Dahlgren and colleagues found lower levels of FSH in 50-year old women with a previous PCOS diagnosis compared with controls, in spite of the fact that the women with PCOS had been wedge-resected [61].

Inhibin B
Inhibin B is a dimeric non-steroidal glycoprotein produced by granulosa cells, which inhibits production and/or release of FSH in the pituitary gland [62]. In normal ovulatory cycles the serum concentration of inhibin-B is inversely correlated with FSH concentrations and increases up to the midpoint of the follicular phase, when it reaches a maximum peak together with the mass of granulosa cells. Thereafter, the concentration decreases progressively and remains low in the luteal phase, except for a brief new elevation after the LH surge [63]. Findings on inhibin B and ovarian reserve are more discrepant [64, 65] and its use is limited, presumably because of strong menstrual cycle-dependency.

Number of antral follicles and ovarian volume
Polycystic ovaries are larger and contain more antral follicles, as assessed by transvaginal ultrasound [66, 67]. An important biopsy study indicated a much increased density of follicles at primary stages in polycystic ovaries compared with normal ovaries, suggesting that women with polycystic ovaries may actually be endowed with a larger ovarian reserve at birth [34].

Metabolic consequences of PCOS
PCOS is associated with insulin resistance and hyperinsulinism, type 2 diabetes, dyslipidemia and possibly cardiovascular disease [68].

Insulin resistance
Studies over the past two decades have indicated that insulin resistance is a pathogenic characteristic feature of the polycystic ovary syndrome, particularly in obese individuals [69].

Insulin resistance, i.e. impaired stimulation of glycogen formation in all major target tissues, such as skeletal muscle, liver, kidney, and adipose tissue, and dysfunction of beta-cells, i.e. inability to compensate for insulin resistance, characterize type 2 diabetes [70]. Approximately 40 % of newly diagnosed type 2 diabetes subjects already suffer from clinical macroangiopathy at the time of diagnosis [71]. Thus, health care providers and groups at risk should be aware of the fact that PCOS, which is a pre-diabetic state, is not harmless. Beck-Nielsen and Groop presented the following model for the development of type 2 diabetes: Insulin resistance is the trigger which increases the insulin demands thereby unmasking the
defect in the beta-cell. A normal beta-cell may compensate for the insulin resistance by increasing the insulin secretion while the dysfunctional beta-cell in the long term can not, thus resulting in hyperglycemia [71]. Insulin secretion, especially first phase, tends to be increased rather than decreased in the pre-diabetic phase and is appropriate for (i.e. correlated with) the level of insulin resistance [72].

Insulin resistance in women with PCOS is closely associated with abdominal obesity and hyperandrogenism [73-76] at a lower BMI than healthy controls [77, 78]. The heterogeneous nature of the PCOS population is, however, reflected by more conflicting data on insulin sensitivity in lean women with PCOS, where some studies indicate that they have reduced insulin sensitivity [79-83], whereas others fail to find differences in insulin sensitivity between lean women with PCOS and weight-matched controls [84-87]. Some studies indicate that patients without hyperandrogenic features do not develop insulin resistance [88, 89].

The diagnosis of insulin resistance is based on results of tolerance test such as the oral glucose tolerance test (OGTT), the euglycemic-hyperinsulinemic glucose clamp, or calculations based on peripheral basal fasting insulin and glucose such as the homeostatic model assessment of IR (HOMA-IR) [90], the quantitative insulin sensitivity check index (QUICKI) [91], or the Matsuda insulin sensitivity index, calculated from OGTT values [92]. The Matsuda index represents both hepatic and peripheral tissue sensitivity to insulin and is highly correlated with insulin sensitivity parameters of the euglycemic-hyperinsulinemic glucose clamp [92].

Several serum markers have been tested in order to simplify the diagnosis of insulin resistance. For instance, hyperinsulinemia results in increased circulating levels of free IGF-1 and reduction in IGFBP-3 [93]. Both insulin and IGF-1 receptor genes are expressed in human thecal cells and can stimulate steroid production there [94]. Free IGF-1 is a potent regulator of cell growth [95]. The actions of IGF-I is regulated by six circulating bindings proteins [96]. IGFBP-3 has been demonstrated in follicular fluid of normally cycling women and increasing FSH levels during maturation of a dominant follicle inhibits basal secretion of IGFBP-3 [97].

**Early insulin response**

Early insulin response is an expression of pancreatic beta-cell function. Beta-cell function can be estimated by the early phase insulin secretion in oGTT (expressed as the insulinogenic index) or by the intravenous glucose tolerance test (IVGTT) (expressed as the insulin peak, insulin increment, areas under the curves (AUC) for insulin and glucose) [98].

The Botnia clamp is an intravenous glucose tolerance test followed by a euglycemic-hyperinsulinemic clamp and provides reliable and independent measures of insulin sensitivity and beta-cell function during the same test.
Studies in Pima Indians have revealed lowered insulin secretion in those with impaired glucose tolerance (IGT) or type 2 diabetes, but exaggerated early insulin secretion in Indians with normal glucose tolerance [100]. Similar results were obtained in PCOS women [85]. In the long-term follow-up of the off-springs of parents with type 2 diabetes, subjects who developed type 2 diabetes within a 10-20-year period presented with higher insulin responses to glucose, especially first phase of insulin secretion, compared with subjects who continued to be normoglycemic [72]. Thus, an enhanced early insulin response, which is not fully explained by insulin resistance, may be a feature of subjects prone to insulin resistance and type 2 diabetes [72, 85, 100].

**Diabetes in women with PCOS**

Prevalence rates for glucose intolerance are reported to vary between 38 - 45 % in younger women with PCOS but these studies have included subjects who predominantly are overweight or obese [101, 102]. In lean women with PCOS, approximately 12 % are reported to display glucose intolerance (IGT or type 2 diabetes) [102]. In PCOS women from the Mediterranean region, 15.7 and 2.5% displayed impaired glucose tolerance and type 2 diabetes, respectively [103].

**Metabolic disturbances in PCOS women**

The metabolic syndrome (MetS) is a cluster of metabolic disturbances that increases the risk of cardiovascular disease (CVD) and diabetes. The metabolic syndrome has several definitions and should not be regarded as a syndrome per se. According to a scientific statement of the American Heart Association (AHS) and the US National Cholesterol Education Program/Adult Treatment Panel III (NCEP/ATPIII), the metabolic syndrome is present if three of following five criteria are fulfilled: elevated waist circumference (≥ 88 cm), elevated triglycerides (≥ 1.7 mmol/L or on drug treatment for elevated triglycerides), reduced high density lipoprotein cholesterol (HDL) (< 1.1 mmol/L or on drug treatment for reduced HDL), elevated blood pressure (≥ 130 mm Hg systolic blood pressure or ≥ 85 mm Hg diastolic blood pressure or on antihypertensive drug treatment in a patient with a history of hypertension), and elevated fasting glucose (≥ 5.6 mmol/L or on drug treatment for elevated glucose) [104]. Prevalence of the metabolic syndrome in PCOS women vary between 11 to 47.3 % in various studies [68, 105-111]. A considerable portion of women with PCOS fulfill the criteria for the metabolic syndrome in view of a higher reported incidence of hypertension, dyslipidemia, visceral obesity, insulin resistance and hyperinsulinemia in this population [106].
Ambulatory day-time systolic and mean arterial blood pressures has been described to be higher in women with PCOS compared with women with normal ovaries and regular menstruation, even when adjusted for BMI [112, 113]. Abnormal lipid profile was observed in 70% of PCOS women [114], including elevated levels of cholesterol, triglycerides, and low-density lipoprotein cholesterol (LDL) and reduced levels of HDL-cholesterol and apolipoprotein A-1 [113, 115, 116]. Obesity has an important influence on the lipid profile [22, 117], but also lean PCOS women have been reported to display abnormal lipid levels [118]. Waist circumference is the best anthropometric correlate of the amount of visceral adipose tissue and is advised to be used in the assessment of cardiovascular risks [119] and insulin resistance [120].

Cardiovascular consequences of PCOS

Cardiovascular disease is the leading cause of death in developed countries [121]. Most cardiovascular events in women are caused by coronary heart disease (CHD) that is most commonly presented by angina pectoris [122]. The major identified risk factors for CHD in women are tobacco use, hypertension, diabetes mellitus, dyslipidemia with elevated levels of fasting total cholesterol, and LDL, as well as low levels of HDL, obesity, particularly of abdominal type, sedentary lifestyle, and atherogenic diet [123].

Multiple studies have indicated that women with polycystic ovary syndrome have several risk factors for cardiovascular disease, including centripetal obesity, hypertension, type 2 diabetes mellitus and dyslipidemia [124-128]. However, it remains unclear whether women with PCOS actually experience an increased number of cardiovascular events compared to an appropriate reference population [129].

Endothelial function in women with PCOS

Endothelial dysfunction is an early event in the development of atherosclerosis, preceding plaque formation and clinical disease. Endothelial function has been evaluated in PCOS patients, mostly by measurement of post-ischemic flow mediated dilatation of the brachial artery with high-resolution ultrasonography but also by measurements of arterial stiffness using pulse wave velocity [130-136]. However, these studies have mainly been conducted in young patients with PCOS and no long-term follow up studies on endothelial function in older PCOS patients have been conducted.
Prior long-term studies in women with PCOS

**Reproductive outcome**

Very few studies assessing the long-term reproductive outcome and ovarian reserve in older women with previously confirmed PCOS have been conducted. The typical ultrasound features of PCOS appear to diminish with increasing age. In a Finnish population-based cross-sectional study, polycystic ovaries were prevalent in 6% of healthy women 34 years of age and in 10% of women of similar age who fulfilled criteria for the metabolic syndrome. In women 44 years of age, the prevalence of polycystic ovaries was 2% and 5% in healthy controls and patients with the metabolic syndrome, respectively [137].

Although studies on reproductive performance in older PCOS patients are few, some studies have indicated that menstrual cycles are normalized with increasing age, at least in some of the patients [61, 138]. This also holds true for subjects who have not undergone ovarian wedge resection [61].

**Cardiovascular disease**

Two relatively small long-term follow-up studies of PCOS patients with prior history of ovarian wedge resection have indicated that these patients suffer an increased risk of myocardial infarction during the menopause [139, 140] and that they have an increased prevalence of coronary artery disease already in the perimenopausal period [140]. Register-based long-term follow-up studies have indicated an increased prevalence of cerebrovascular disease [141], however, morbidity and mortality from coronary heart disease among women with PCOS was not as high as previously predicted [141, 142]. More recently, postmenopausal women with a retrospective diagnosis of PCOS were reported to have more angiographic coronary artery disease and a lower cumulative 5-year cardiovascular event-free survival compared to women without clinical features of PCOS [143]. Furthermore, postmenopausal mothers of PCOS patients were demonstrated to have a higher prevalence of cardiovascular events than controls [144].

**Insulin resistance and diabetes**

The, above cited, long-term follow-up studies of women with PCOS also indicated an increased risk of type 2 diabetes in the menopause [61, 140]. Longitudinal studies of women with PCOS with focus on age-related changes in plasma androgens, insulin sensitivity and insulin secretion are lacking. Also, no studies on the long-term risks for developing type 2 diabetes and impaired glucose tolerance among PCOS patients diagnosed according to Rotterdam criteria have been performed.
AIMS

The aims of this thesis were:

to describe the reproductive outcome in middle-aged women with a previous diagnosis of PCOS, both in terms of live birth rate, pregnancies and of miscarriages (paper I);

to assess the remaining ovarian reserve in middle-aged women with a previous diagnosis of PCOS compared with age-matched controls (paper I);

to investigate pulse wave reflection which depends on stiffness in the aorta and resistance in lower extremities, and endothelial function in resistance arteries in women previously diagnosed with PCOS in comparison to age-matched healthy controls (paper II);

to examine changes over time in plasma androgen concentrations, early insulin response and insulin sensitivity of women with a previous diagnosis of PCOS (paper III);

to examine glucose tolerance and insulin sensitivity in middle-aged women previously diagnosed with PCOS in comparison to age-matched healthy controls (paper IV).
Material and methods

Patients

PCOS diagnosis

Eligible patients were identified by the out-patient register at Uppsala University Hospital. Inclusion criteria for the study were diagnosis of polycystic ovary syndrome between 1987 and 1995 and age at the follow-up > 35 years. As the emphasis in this study was to follow patients with a PCOS diagnosis made by transvaginal ultrasound the medical records at the time of diagnosis were screened to confirm the diagnosis and to verify the use of transvaginal ultrasound for the diagnosis. Only subjects with a diagnosis of PCOS according to the Rotterdam criteria [7] were included and one of the features had to be polycystic ovaries on ultrasound examination [59, 66]. In addition to the ultrasound criterion, one of the following two features had to be present for the PCOS diagnosis 1) oligomenorrhoea, with eight or fewer menstruations in the previous 12 months, or amenorrhea 2) clinical and/or biochemical signs of hyperandrogenism such as testosterone > 2.7 nmol/l, elevated dehydroepiandrosterone (DHEAS), free androgen index (FAI) > 5.0, or hirsutism (> 7 on the Ferriman and Gallway scale). The PCOS diagnosis also implied that no evidence of thyroid disease (normal thyroid-stimulating hormone (TSH)), adrenocortical dysfunction (normal 17-hydroxyprogesterone), or hyperprolactinemia (prolactin < 30 μg/l) was present at diagnosis. Subjects with a PCOS diagnosis based on clinical history and laboratory findings but without transvaginal ultrasound examination were not included.

Healthy controls were selected from population registrars. For each PCOS patient, three healthy controls residing in Uppsala County and born during the same month as the index patients were selected. The control women were invited by letter, and in case the first control did not respond to the invitation, the second (and third) control subject was subsequently invited. Healthy control status was assured by absence of polycystic ovaries on transvaginal ultrasound. Furthermore all control subjects denied a prior history of oligomenorrhoea or amenorrhea (lasting more than three months).
The patients and controls gave a written informed consent and the Independent Ethical Review Board at Uppsala University, Sweden approved the study.

In all, 174 PCOS patients had been previously identified with a PCOS diagnosis. Of these, three patients (1.7%) were dead, 12 patients had emigrated (6.9 %), and 14 patients (8.0 %) were living too far from Uppsala to be invited. Hence 145 patients (83.3 % of the identified patients) were invited to participate in the study.

Among the 145 invited PCOS patients, 84 women (57.9 %) attended the clinical examination and seven women (4.8 %) filled out the questionnaires but were unable to visit the clinic. Among PCOS patients still living in the Uppsala area 61 women (66.3 %) attended the clinical examination and three patients (3.2 %) filled out the questionnaires, whereas the corresponding figures for PCOS patients living outside the Uppsala area were 23 (43.4%) and four (7.5 %), respectively. At the time of the original diagnosis, 58 (40.0 %) of invited PCOS patients had PCO and oligomenorrhea only, 8 (5.5 %) had PCO and hirsutism only, and 79 (54.5 %) had PCO, oligomenorrhea and hirsutism. Eighty-seven age-matched control women participated in the study.

The number of patients included in the four studies is described in figure 2. In the first paper all women, including those who only filled out the questionnaire were included. In paper II, only 67 PCOS patients participated in the pulse wave analyses due to logistic reasons. According to the protocol, hyperinsulinemic euglycemic clamp or oral glucose tolerance test should be performed in addition to the pulse wave analyses but these measurements had to be performed on different days. Both analyses required that patients should be fasting, and terbutaline administration results in increased blood glucose levels. For patients who needed to travel great distances it was thus not possible to perform all measurements. In essence, the 67 women who participated in this sub-study were the ones who could come in to the clinic twice, i.e. women still residing in Uppsala County. In paper III, only women who previously had been investigated with hyperinsulinemic euglycemic clamp and intravenous glucose tolerance test were included. Finally, in paper IV all women who came to the health care investigation were included.

There were no differences in parity between participating and non-participating PCOS patients, for details see paper I. Also, no differences in metabolic variables were found between patients who participated and those who did not participate in paper III.
Design

Paper I
Long-term follow-up study describing the reproductive outcome in middle-aged women with a previous diagnosis of PCOS, both in terms of live birth rate, pregnancies, and miscarriages. A secondary aim was to assess the remaining ovarian reserve in women with PCOS compared with age-matched controls.

Paper II
Long-term follow-up study investigating pulse wave reflection (baseline aortic augmentation index (AI)) which depends on stiffness in aorta and resistance in lower extremities, and endothelial function in resistance arteries (expressed as change in radial AI after terbutaline provocation) in women previously diagnosed with PCOS in comparison to age-matched healthy controls, randomly selected from the general population.

Paper III
Long-term follow-up study examining changes over time in plasma androgen concentrations, early insulin response and insulin sensitivity in women with a previous diagnosis of PCOS.
Methods

All subjects consenting to participate attended a health examination at the department of Women’s and Children’s Health, Uppsala University Hospital. As the reproductive status was variable among subjects (premenopausal with or without hormonal contraception, postmenopausal with or without hormone therapy (HT), the visit was not scheduled according to menstrual cycle phase. However, records were kept on last menstrual period and transvaginal ultrasound examination together with assays of estradiol, FSH and LH confirmed the cycle phase in subjects without hormonal contraception.

All blood samples for the endocrine measurements were obtained in a standardized manner between 08.00 h – 09.00 h during rest in the supine position and after an overnight fast.

Subjects were weighed on an electronic scale. The subjects wore light clothes and no shoes and weight was measured to the nearest 0.2 kg. Height without shoes was measured to the nearest centimeter. Body mass index was calculated as weight (kg) divided by height (m²). Waist circumference was measured midway between the lower rib margin and iliac crest. The hip was measured at the maximum circumference over the buttocks and to the nearest 0.0 or 0.5 cm. Blood pressure was measured twice in every person after a five-minute rest in a sitting position with digital manometer. The mean value of the two measurements was used in the study.

The free androgen index was calculated from testosterone/SHBG x 100.

Paper I

The ultrasound examination

The ultrasound examination was performed with a 7 MHz transvaginal probe by either of two physicians (Miriam Hudecova and Inger Sundström Poromaa). The ultrasound measurements were obtained in real-time according to a standardized protocol, with examination of the ovaries made under the highest possible magnification. After the longest medial axis of the ovary had been determined, the second dimension was measured, and then
the vaginal probe was rotated 90 degrees to obtain the third dimension. Ovarian volume was calculated in the largest ovary according to a simplified formula for an ellipsoid (0.523 x length x width x thickness [66]. The ovary was scanned in both longitudinal and transverse cross-section from the inner to the outer margins to count the total number of follicles. All follicles, antral and growing, were counted.

**Questionnaire**
The participants filled out a questionnaire on reproductive health, including obstetric history (previous pregnancies, previous live births), infertility history (duration of infertility, infertility treatments and infertility treatment in relation to every live birth), menstrual cycle history (menstrual frequency during the last 12 months), treatment, and ongoing hormonal treatment (any treatment with ovarian steroids such as oral contraceptives, progestagens, hormonal IUD, postmenopausal hormone therapy).

**Assays**
Serum concentrations of testosterone, SHBG, FSH, LH were analysed by solid-phase chemiluminescent immunometric assays and estradiol was measured by competitive immunoassay, using commercial kits obtained from Siemens Medical Solutions, Germany. Detection limits for the estradiol essays was 73.0 pmol/l and for testosterone 0.5 nmol/l. Total coefficients of variation varied between 5.7 and 10.6% for these analyses. The serum concentrations of AMH were determined using enzymelinked immunoassay kits, from Immunotech Beckman Coulter Company, France. The detection limit for AMH was 0.07 pmol/l and levels below this limit were considered undetectable. Total coefficient of variation was 12.3% for the AMH analyses.

**Paper II**

**Pulse wave analyses**
Endothelium-dependent vasodilation (EDV) in resistance arteries was evaluated by aplanation tonometry of the radial artery, where the peripheral pulse pressure waveform in response to adrenergic β-2 receptor agonist stimulation may be captured and used as a valid measure of EDV [145, 146]. This method has been thoroughly evaluated and a reduced response to β-2 agonists has been demonstrated in patients with insulin resistance, hypercholesterolemia, coronary heart disease, diabetes mellitus, and the metabolic syndrome [146-149].

A micromanometer tipped probe (Sphygmocor, AtCor Medical Pty Ltd, West Ryde, Sydney, Australia) was applied to the skin surface overlying the radial artery and the peripheral radial pulse wave was continuously recorded. The
mean values of 10 pulse waves were used for analyses. Recordings were regarded as satisfactory if the variations in the systolic peak and the diastolic peak were 5% or below. The maximal systolic peak and the reflected waves were identified by the calculations of the first and second derivative of the pulse curve. After a baseline recording, terbutaline was subcutaneously administered (0.25 mg in the upper part of the arm), and a re-evaluation of the pulse wave was performed after 15 and 20 minutes. The maximal change occurring at either 15 or 20 minutes was used for calculations [145]. Each test was performed and evaluated by two examiners (Miriam Hudecova and Inger Sundström Poromaa) throughout the study. Intra- and interobserver coefficient of variation were 3.4% and 2.3 %, respectively.

For this study we used two measures of augmentation index (AI): 1) AI-radial which is the augmentation index derived from the first reflected wave using the radial artery recording [147], and 2) AI-aorta which corresponds to the augmentation index obtained after transformation of the radial pulse wave to the corresponding central pulse wave by use of a validated transfer function [148]. These variables are reported as baseline values and AI-radial also as relative change from baseline after terbutaline administration. Thus, a large reduction of the pulse wave in response to terbutaline indicates a good response.

Figure 3. Radial and derived aortic pulse waveform obtained from pulse wave analysis. AI-radial = b/a, AI-aortic = b-a/a.

**Assays**

Lipid variables, fasting plasma glucose and fasting insulin were measured by standard laboratory techniques. Serum concentrations of testosterone and SHBG were analyzed by solid-phase chemiluminescent immunometric assays and. Homeostatic Model Assessment of Insulin Resistance Index
(HOMA-IR) was defined as: fasting blood glucose × serum insulin/22.5 [150].

**Paper III**

*Botnia clamp*

At the index assessment, an IVGTT and a hyperinsulinemic euglycemic clamp were performed on two different days. For the present study, the Botnia clamp technique was used. The Botnia clamp is an intravenous glucose tolerance test followed by a euglycemic-hyperinsulinemic clamp and provides reliable and independent measures of insulin sensitivity and beta-cell function during the same test [99]. Furthermore, the Botnia clamp technique provides measures of the early insulin response and insulin sensitivity comparable to measurements made on separate days [99].

After an injection of 300 mg/kg body weight of D-glucose, samples were drawn at -10, 2, 4, 6, 8, 10, 20, 40, 60 and 90 min for analyses of plasma insulin and glucose (time from initiation of the injection of glucose). The insulin peak was calculated as the mean value of the plasma insulin concentrations at 4, 6 and 8 min, and the insulin increment was calculated by subtracting the mean fasting plasma insulin concentrations from the peak value. The AUC for insulin and glucose were calculated as the deviations from the basal value integrated over the sampling time. The IVGTT procedures and calculations were similar to the index assessment [85].

After 90 min, a priming dose of insulin was given followed by an infusion (56 mU/m²) of short-acting human insulin (Actrapid; Novo Nordisk, Bagsvaerd, Denmark) for 120 min. Euglycemia (5.1 mmol/l plasma glucose) was maintained by measuring the plasma glucose every 5 min and adjusting the rate of infusion of a 20% glucose solution accordingly. Blood samples for insulin analyses were drawn at 60, 80 and 120 min after the start of the clamp. The coefficient of variation for the steady-state plasma glucose concentration for a single clamp was 5.5 %. The insulin sensitivity index (M/I) was calculated by dividing the amount of glucose infused during the last 60 min of the clamp with the mean steady state insulin concentration during the same time (milligram per kg body weight⁻¹ min⁻¹ x mU/L x 100). The insulin infusion rate and target plasma glucose during the clamp were similar to the index assessment [85].

**Assays**

Apolipoprotein A-1, apolipoprotein B, triglycerides, total cholesterol, high density lipoprotein cholesterol (HDL-cholesterol), low density lipoprotein cholesterol (LDL-cholesterol), high sensitive C-reactive protein (hs-CRP), and glucose, were analyzed on an Architect ci8200 (Abbott Laboratories, Abbott Park, IL, USA). The total CV were 0.9% at 2.25 g/l for
ApoA-I, 1.2% at 1.73 g/l for Apo B, 1.1% at 0.9 mmol/L, 0.5% at 5.7 mmol/L for total cholesterol, 2.3% at 1.0 mmol/L for HDL-cholesterol, 1.0% at 2.0 mmol/L for LDL-cholesterol, for triglycerides, 0.8% at 8 mg/L for hs-CRP, and 1.0% at 4.4 mmol/L for glucose.

Insulin, C-peptide and sex hormone binding globulin (SHBG) were analyzed on Modular E170 (Roche Diagnostics, Mannheim, Germany). The total imprecision of the methods, expressed as coefficient of variation (CV), were 1.6% at 7.0 mU/L for insulin, 1.5% at 43 nmol/L for SHBG, and 1.5% at 0.45 nmol/L for C-peptide. The analytical method for SHBG was changed in the time-interval between assessments, resulting in 13 % higher values at the follow-up investigation. For statistical comparisons, the SHBG values at the index assessment were transformed using a correction factor of 1.13. The SHBG values from the index assessment which are presented in text and tables were also transformed.

Testosterone was analysed by the same radioimmunoassay at both occasions (Diagnostic Products Corp., Los Angeles, CA). Within- and between-assay coefficients of variation for testosterone were 5.5 % and 7.0 %.

HOMA-IR was defined as: fasting blood glucose × serum insulin/22.5 [150].

Paper IV

Oral glucose tolerance test
With the exception of women with previously known diabetes and women who were subjected to euglycemic hyperinsulinemic clamp, all women performed a 75-g OGTT after over-night fasting. Blood samples for measurement of serum insulin and plasma glucose were obtained at 0, 30, and 120 minutes.

Assays
Apolipoprotein A-I (ApoA-I), Apolipoprotein B (ApoB), triglycerides, high-sensitivity C-reactive protein (hs-CRP) and glucose, were analyzed on an Architect ci8200 (Abbott Laboratories, Abbott Park, IL, USA). The total imprecision (coefficient of variation, CV) of the instrument for the analytes were 0.9% at 2.25 g/l for ApoA-I, 1.2% at 1.73 g/l for ApoB, 1.1% at 0.9 mmol/L for triglycerides, 0.8% at 8 mg/L for hs-CRP, and 1.0% at 4.4 mmol/L for glucose.

Insulin, C-peptide and SHBG were analyzed on Modular E170 (Roche Diagnostics, Mannheim, Germany), IGFBP-3 was analyzed on an Immulite 2500 (Siemens Healthcare Diagnostics, Deerfield, IL, USA), and proinsulin was analyzed with a sandwich ELISA (Mercodia, Uppsala, Sweden). The total imprecision of the methods were 1.6% at 7.0 mU/L for insulin, 1.5% at
43 nmol/L for SHBG, 1.5% at 0.45 nmol/L for C-peptide, 3.6% at 0.85 mg/L for IGFBP-3, and 4.1% at 21.7 pmol/L for proinsulin.

The Matsuda insulin sensitivity index, which is an index of whole-body insulin sensitivity was calculated according to this formula: \((10,000/\text{square root of [fasting glucose x fasting insulin] x [mean glucose x mean insulin during OGTT]})\) [92].

Beta-cell function was estimated by the early phase insulin secretion (the insulinogenic index) and calculated as \([(30 \text{ min insulin} - \text{fasting insulin}) / 30 \text{ min glucose}]\) [98].

Impaired glucose tolerance and diabetes were defined using World Health Organization criteria.

Results

Paper I

Reproductive outcome

Among PCOS patients, 83 had attempted a pregnancy, 79 had become pregnant, and 72 had given birth at least once. There was no difference between PCOS patients and controls in number of women who had given birth. Among women who had attempted a pregnancy, 72 (86.7 %) of PCOS patients and 76 (91.6 %) of control subjects had given birth to at least one child. The overall rate of subjectively defined spontaneous conception was also high among PCOS patients. Among the 83 PCOS patients who had attempted a pregnancy, 56 (67.5 %) became spontaneously pregnant at least once. Among the PCOS patients who had given birth to a child, 53 (73.6 %) did so at least once following a spontaneous conception. Among PCOS patients who had attempted a pregnancy 63 (75.9%) had sought care for infertility and 56 (67.5%) patients had, at some point, been treated for infertility.

The rate of miscarriages was not increased in PCOS patients (PCOS patients \(0.6 \pm 1.1\) miscarriages vs. \(0.6 \pm 0.9\) miscarriages in control subjects). Among women who conceived at least once, 36 (45.6 %) of PCOS patients and 41 (49.4 %) of control women had at least one miscarriage.

The majority of subjects were still premenopausal (87.9 % of PCOS women and 80.5 % of controls). Pre-menopausal PCOS patients without hormonal contraception reported \(8.8 \pm 3.9\) menstruations during the last year while control women reported \(11.4 \pm 2.2\) menstrual periods during the last year, \(p < 0.01\). The number of premenopausal PCOS patients without...
hormonal contraception who had had eight or more menstrual bleedings during the last year was 38 (73.1%).

**Ovarian reserve**
Mean ovarian volume and number of antral follicles in pre-menopausal PCOS patients without hormonal treatment were significantly higher than in corresponding control subjects, 9.5 ± 6.6 ml compared to 6.6 ± 4.1 ml (p < 0.001) and 11.7 ± 12.4 compared to 5.0 ± 2.4 (p < 0.001), respectively. Mean ovarian volume and number of antral follicles were also higher in pre-menopausal women with PCOS compared with controls, when the comparisons were restricted to women evaluated in the follicular phase, table I.
Table I. Mean ± SEM serum concentrations of ovarian volume, number of antral follicles, ovarian steroids and gonadotropins in 52 premenopausal PCOS patients and 56 premenopausal controls without hormone treatment.

<table>
<thead>
<tr>
<th></th>
<th>Premenopausal women</th>
<th></th>
<th>Premenopausal women in the follicular phase</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>without hormone treatment</td>
<td></td>
<td>follicular phase</td>
<td></td>
</tr>
<tr>
<td></td>
<td>PCOS (n = 52)</td>
<td>Controls (n = 56)</td>
<td>PCOS (n = 16)</td>
<td>Controls (n = 30)</td>
</tr>
<tr>
<td>Age, years</td>
<td>42.4 ± 4.5</td>
<td>41.5 ± 4.6</td>
<td>41.9 ± 3.8</td>
<td>42.7 ± 4.2</td>
</tr>
<tr>
<td>Ovarian volume, ml</td>
<td>9.5 ± 0.9**</td>
<td>6.6 ± 0.5</td>
<td>8.9 ± 1.3</td>
<td>6.5 ± 0.7</td>
</tr>
<tr>
<td>Antral follicles, n</td>
<td>11.7 ± 1.7***</td>
<td>5.0 ± 0.3</td>
<td>10.1 ± 1.2***</td>
<td>5.2 ± 0.4</td>
</tr>
<tr>
<td>Estradiol, pmol/l</td>
<td>514 ± 79*</td>
<td>710 ± 90</td>
<td>522 ± 156</td>
<td>734 ± 134</td>
</tr>
<tr>
<td>Testosterone, nmol/l</td>
<td>1.5 ± 0.1**</td>
<td>1.0 ± 0.1</td>
<td>1.6 ± 0.2*</td>
<td>1.0 ± 0.1</td>
</tr>
<tr>
<td>SHBG, nmol/L</td>
<td>50.4 ± 4.5*</td>
<td>64.0 ± 4.8</td>
<td>52.9 ± 6.9</td>
<td>63.0 ± 5.3</td>
</tr>
<tr>
<td>Free androgen index</td>
<td>5.1 ± 0.8**</td>
<td>2.0 ± 0.2</td>
<td>4.6 ± 1.2</td>
<td>2.0 ± 0.3</td>
</tr>
<tr>
<td>FSH, IU/L</td>
<td>6.2 ± 1.1*</td>
<td>9.6 ± 1.3</td>
<td>6.2 ± 0.8</td>
<td>9.7 ± 1.4</td>
</tr>
<tr>
<td>LH, IU/L</td>
<td>7.1 ± 0.7</td>
<td>10.4 ± 1.7</td>
<td>7.5 ± 1.1</td>
<td>12.1 ± 2.8</td>
</tr>
<tr>
<td>AMH, pmol/l</td>
<td>39.9 ± 6.1***</td>
<td>15.7 ± 2.1</td>
<td>37.2 ± 7.9***</td>
<td>9.9 ± 1.9</td>
</tr>
</tbody>
</table>

*p < 0.025 in comparison with respective control group, Mann-Whitney U test.

**p < 0.01 in comparison with respective control group, Mann-Whitney U test.

***p < 0.001 in comparison with respective control group, Mann-Whitney U test.

Free androgen index was increased in PCOS patients compared to their age-matched controls. AMH serum concentrations were significantly elevated in women with PCOS, also in the subgroup of women who were evaluated in the follicular phase. Premenopausal PCOS patients had lower levels of FSH and estradiol than control subjects, table I.

Ovarian volume and number of antral follicles were significantly correlated with serum concentrations of AMH, but not to serum concentrations of FSH or estradiol, table II.
Table II. Correlation between ovarian volume and follicle count and reproductive hormones in 52 premenopausal PCOS patients and 56 premenopausal control subjects without hormone treatment.

<table>
<thead>
<tr>
<th>Ovarian volume, ml</th>
<th>Follicles count</th>
<th>E2</th>
<th>T</th>
<th>SHBG</th>
<th>FAI</th>
<th>FSH</th>
<th>AMH</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.62**</td>
<td>0.09</td>
<td>0.11</td>
<td>-0.10</td>
<td>0.17</td>
<td>0.03</td>
<td>0.35**</td>
<td></td>
</tr>
<tr>
<td>Antral follicles, n</td>
<td>-0.10</td>
<td>0.09</td>
<td>-0.18</td>
<td>0.19</td>
<td>-0.15</td>
<td>0.54**</td>
<td></td>
</tr>
<tr>
<td>Estradiol</td>
<td>0.04</td>
<td>0.04</td>
<td>-0.08</td>
<td>0.06</td>
<td>-0.12</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Testosterone</td>
<td>-0.22*</td>
<td>0.80***</td>
<td>-0.17</td>
<td>0.25**</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SHBG</td>
<td>-0.53***</td>
<td>-0.03</td>
<td>-0.20</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FAI</td>
<td>-0.17</td>
<td>0.30**</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FSH</td>
<td>-0.22*</td>
<td>0.30**</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LH</td>
<td>-0.04</td>
<td>0.30**</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

T = testosterone, E2 = estradiol, *p < 0.025 Pearson’s Correlation Coefficient, **p < 0.01 Pearson’s Correlation Coefficient, ***p < 0.001 Pearson’s Correlation Coefficient

The relationship between ovarian reserve, PCOS status and age is depicted in figure 2 in paper I. Multiple regression analyses indicated that ovarian volume, number of antral follicles and AMH serum concentrations declined with age in both groups (p < 0.001, respectively) and that PCOS patients had higher values for the above measures across ages compared to controls (p < 0.001 for each ovarian reserve variable). Covariance analyses indicated no group by age interactions for the ovarian reserve measures, indicating that the age-related decline was similar between groups (ovarian volume F(1,121) = 0.76; number of antral follicles F(1,121) = 1.13: AMH F(1,123) = 1.74; p = 0.055)

Paper II

In all, 67 PCOS patients and 66 control subjects were included in the study. In two PCOS patients and two control subjects, satisfactory pulse wave recordings could not be obtained. In the remaining subjects, the
software did not properly recognize either AI-radial or AI-aorta in three control subjects.

At the index assessment 32 (47.8 %) of patients presented with PCO and oligomenorrhea, 4 (6.0 %) had PCO and hyperandrogenism, and 31 (46.3 %) had PCO, oligomenorrhea, and hyperandrogenism. At the follow-up investigation 22 (32.8 %) patients still fulfilled and 27 patients (40.3 %) no longer fulfilled diagnostic criteria for PCOS. In 18 patients (26.9 %) PCOS criteria could not be assessed because of postmenopausal status, prior surgery or ongoing use of hormonal contraception.

Analysis of basic characteristics and major cardiovascular risk factors at the follow-up investigation revealed that PCOS patients had significantly increased weight, BMI, waist circumference and waist:hip ratio in comparison to control subjects. Likewise, systolic and diastolic blood pressures, heart rate, HOMA-IR and triglycerides were higher in PCOS patients than in control subjects.

None of the PCOS patients or control subjects reported prior cardiovascular events such as nonfatal myocardial infarction, coronary revascularization, nonfatal stroke, or coronary disease. There were no significant differences in frequency of family history of myocardial infarction or stroke, but prevalence of diabetes was twice as high in first-degree relatives of patients with PCOS as in those of control subjects.

**Pulse wave analyses**

There was no difference in baseline AI-aorta between PCOS patients and control subjects, table III. Baseline AI-radial was increased and percent decrease in AI-radial following terbutaline administration was less pronounced in PCOS patients in comparison with control subjects, table III. The difference in the change in AI-radial following terbutaline administration remained when adjusted for use of combined oral contraceptives/HRT as well as postmenopausal status ($\beta = 0.197$, $p < 0.05$), and smoking ($\beta = 0.191$, $p < 0.05$). However, after adjustment for BMI, none of these differences between PCOS patients and healthy controls remained, table IV. The strongest independent explanatory factor for the variability in endothelial function was HOMA-IR, table IV.
Table III. Mean ± SD for Pulse Wave Analysis Variables in PCOS Patients, Subgroups of PCOS Patients, and Control Subjects

<table>
<thead>
<tr>
<th></th>
<th>All PCOS patients (n = 65)</th>
<th>PCOS patients with persisting symptoms at follow-up investigation (n = 21) (^a)</th>
<th>PCOS patients with resolved symptoms at follow-up investigation (n = 26) (^a)</th>
<th>PCO and oligomenorrhoea at index assessment (n = 32)</th>
<th>PCO, oligomenorrhoea and hirsutism at index assessment (n = 29) (^a)</th>
<th>Control subjects (n = 64)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>BMI</strong></td>
<td>27.4 ± 5.5 (^b)</td>
<td>27.3 ± 5.9 (^b)</td>
<td>25.9 ± 4.8</td>
<td>26.3 ± 3.3</td>
<td>28.5 ± 5.4 (^b)</td>
<td>25.5 ± 3.6</td>
</tr>
<tr>
<td><strong>Baseline AI-radial, %</strong></td>
<td>84 ± 14 (^c)</td>
<td>81 ± 14</td>
<td>84 ± 10</td>
<td>86 ± 12</td>
<td>80 ± 16</td>
<td>81 ± 15</td>
</tr>
<tr>
<td><strong>Baseline AI-aorta, %</strong></td>
<td>27 ± 9</td>
<td>25 ± 9</td>
<td>26 ± 8</td>
<td>29 ± 8</td>
<td>26 ± 9</td>
<td>25 ± 10</td>
</tr>
<tr>
<td><strong>% change AI-radial</strong></td>
<td>-22 ± 14 (^c)</td>
<td>-21 ± 10 (^b)</td>
<td>25 ± 18</td>
<td>-23 ± 13</td>
<td>-21 ± 16</td>
<td>-28 ± 17</td>
</tr>
</tbody>
</table>

\(^a\) Discrepancies in numbers, in comparison to the text, is because satisfactory pulse wave recordings could not be obtained in two PCOS patients

\(^b\) Significantly different from control subjects, one-way ANOVA with Tukey Honestly Significance Difference post hoc test, \(p < 0.05\).

\(^c\) Significantly different from control subjects in unadjusted analyses, \(p < 0.05\). Following adjustment for BMI, none of these differences remained.
Table IV. Results of a stepwise multivariate linear regression analysis of variables associated with percent change in AI-radial following terbutaline.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Model 1</th>
<th>Model 2</th>
<th>Model 3</th>
<th>Model 4</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\beta$</td>
<td>$\beta$</td>
<td>$\beta$</td>
<td>$\beta$</td>
</tr>
<tr>
<td>$R^2$</td>
<td>.04</td>
<td>.13</td>
<td>.18</td>
<td>.23</td>
</tr>
<tr>
<td>PCOS diagnosis</td>
<td>0.19*</td>
<td>0.14</td>
<td>0.14</td>
<td>0.12</td>
</tr>
<tr>
<td>Free androgen index</td>
<td>0.04</td>
<td>-0.03</td>
<td>-0.08</td>
<td>-0.06</td>
</tr>
<tr>
<td>Age</td>
<td>0.01</td>
<td>0.10</td>
<td>0.07</td>
<td></td>
</tr>
<tr>
<td>BMI</td>
<td>0.29**</td>
<td>0.13</td>
<td>0.07</td>
<td></td>
</tr>
<tr>
<td>HOMA-IR</td>
<td></td>
<td>0.29*</td>
<td>0.31*</td>
<td></td>
</tr>
<tr>
<td>Smoking</td>
<td></td>
<td></td>
<td></td>
<td>0.15</td>
</tr>
<tr>
<td>Current treatment for diabetes,</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>hypertension or hyperlipidemia</td>
<td></td>
<td></td>
<td></td>
<td>0.14</td>
</tr>
</tbody>
</table>

Free androgen index, age, BMI and HOMA-IR entered as continuous variables.

$\beta$ standardised regression coefficient,

**Pulse wave analyses in relation to PCOS phenotype at index assessment**

There were no significant differences in the pulse wave variables between PCOS patients who had PCO and oligomenorrhoea and those who had PCO, oligomenorrhoea, and hyperandrogenism at the index assessment, nor between any of these subgroups and control subjects, table III.

**Pulse wave analyses in relation to PCOS phenotype at follow-up investigation**

There was no difference in pulse wave variables between PCOS patients who still fulfilled PCOS criteria at the time of the follow-up and those who no longer did, table 3. However, percent reduction in AI-radial following
terbutaline administration was significantly lower in PCOS patients with persisting symptoms in comparison to control subjects, table 3. Again, this finding did not remain when adjusted for BMI, table III.

Paper III

In all, 73 women with PCOS had participated in the first study using IVGTT and hyperinsulinemic, euglycemic clamp between 1987 and 1995. Of these, 13 subjects were lost to follow up because personal data from the initial study was insufficient for tracing or because they had emigrated or lived too far away from the Uppsala region. Hence, 60 subjects were invited for the follow-up study. Of these, 30 (50.0 %) consented to participate in the Botnia clamp investigation, whereas four subjects only consented to blood sampling. Women who consented to participate in the follow-up investigation and non-participants did not differ on any of the metabolic measures at the index assessment, see paper III for details.

One PCOS patient had developed diet-treated type 2 diabetes since the index assessment and one had an elevated fasting glucose. As results were not affected by the exclusion/inclusion of these two subjects, they were kept in the study. Three women were treated with combined oral contraceptives and one postmenopausal woman was on hormone replacement therapy. These subjects were excluded from analyses of plasma testosterone and SHBG. At the index assessment the distribution of lean, overweight and obese subjects were 15 (44.1 %), 10 (29.4 %) and 9 (26.5%), whereas corresponding figures at the follow-up investigation were 12 (34.3%), 6 (17.1%) and 17 (48.6%), respectively.

At the follow-up investigation 10 (29.4 %) women still fulfilled and 11 women (32.4 %) no longer fulfilled diagnostic criteria for PCOS. In 13 women (38.2 %) PCOS criteria could not be assessed because of postmenopausal status, prior surgery or ongoing use of hormonal contraception.

Metabolic and anthropometric variables over time

Over time, women with PCOS displayed increased BMI, increased plasma SHBG concentration and increased diastolic blood pressure. In contrast, serum testosterone concentrations and free androgen index decreased from the time of index assessment to the follow-up investigation. There were no differences in systolic blood pressure or waist-hip ratio between the two time-points of assessments.
**Insulin sensitivity and acute insulin response over time**

Insulin sensitivity expressed as M/I remained unaltered over time, table V. Similarly, insulin increment was unaltered between the two assessments. Other variables reflecting the early insulin response and glucose-stimulated insulin secretion, such as the insulin peak and AUC insulin decreased over time, table V. These results remained when the two subjects with type 2 diabetes were excluded from the analysis (data not shown). Also, there was no difference in M/I or insulin increment between women with resolved or unresolved PCOS (M/I; resolved PCOS 6.7 ± 2.3 vs. unresolved PCOS 7.6 ± 2.8, insulin increment; resolved PCOS 34.1 ± 20.0 mU/l vs. unresolved PCOS 48.4 ± 19.4 mU/l).

### Table V. Difference in Botnia clamp parameters in PCOS patients between initial and follow-up evaluation

<table>
<thead>
<tr>
<th>Parameter</th>
<th>At diagnosis</th>
<th>Follow-up</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mean ± SD</td>
<td>mean ± SD</td>
</tr>
<tr>
<td>M/I_{60-120}</td>
<td>6.7 ± 3.4</td>
<td>6.3 ± 2.6</td>
</tr>
<tr>
<td>Baseline insulin, mU/lc</td>
<td>11.0 ± 8.1</td>
<td>9.5 ± 7.9</td>
</tr>
<tr>
<td>Baseline glucose, mmol/lc</td>
<td>4.7 ± 0.6</td>
<td>4.8 ± 0.6</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>2.4 ± 2.0</td>
<td>2.1 ± 1.9</td>
</tr>
<tr>
<td>Insulin peak, mU/l</td>
<td>78.1 ± 55.7</td>
<td>60.2 ± 62.0a</td>
</tr>
<tr>
<td>Insulin increment, mU/l</td>
<td>67.7 ± 52.0</td>
<td>51.9 ± 59.4</td>
</tr>
<tr>
<td>AUC insulin</td>
<td>2425 ± 1711</td>
<td>1823 ± 1711a</td>
</tr>
<tr>
<td>AUC glucose</td>
<td>281 ± 62</td>
<td>370 ± 118a</td>
</tr>
</tbody>
</table>

*a* Difference across time, *p* < 0.05 – 0.01

*c* Data available in 34 PCOS patients
Insulin sensitivity at the follow-up investigation in relation to characteristics at the index assessment

Comparisons of endocrine, metabolic and anthropometric variables at the index assessment, depending on current insulin sensitivity, are presented in table VI. PCOS patients who had M/I-levels in the lowest quartile at the follow-up investigation had already at the index assessment increased BMI, increased waist, higher plasma LDL-cholesterol and higher fasting plasma

Table VI. Differences in metabolic variables at baseline or across time between PCOS patients with insulin resistance or intermediate/normal insulin sensitivity at the follow-up.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Lowest M/I quartile at $t_2$</th>
<th>Remaining M/I quartiles at $t_2$</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age at $t_1$</td>
<td>30.0 ± 7.0</td>
<td>25.7 ± 6.3</td>
<td>ns</td>
</tr>
<tr>
<td>BMI at $t_1$, kg/m$^2$</td>
<td>29.2 ± 2.8</td>
<td>25.4 ± 5.2</td>
<td>0.05</td>
</tr>
<tr>
<td>ΔBMI over time, kg/m$^2$</td>
<td>4.8 ± 2.8</td>
<td>1.4 ± 3.9</td>
<td>0.05</td>
</tr>
<tr>
<td>Waist at $t_1$, cm</td>
<td>92.7 ± 10.7</td>
<td>83.3 ± 12.8</td>
<td>0.05</td>
</tr>
<tr>
<td>FAI at $t_1$</td>
<td>19.2 ± 13.4</td>
<td>16.3 ± 13.6</td>
<td>ns</td>
</tr>
<tr>
<td>Testosterone at $t_1$, nmol/l</td>
<td>3.0 ± 1.5</td>
<td>2.8 ± 1.1</td>
<td>ns</td>
</tr>
<tr>
<td>SHBG at $t_1$, nmol/l</td>
<td>17.7 ± 3.6</td>
<td>23.3 ± 13.6</td>
<td>ns</td>
</tr>
<tr>
<td>Total cholesterol at $t_1$, mmol/l</td>
<td>5.2 ± 0.6</td>
<td>4.6 ± 0.7</td>
<td>ns</td>
</tr>
<tr>
<td>HDL-cholesterol at $t_1$, mmol/l</td>
<td>1.0 ± 0.2</td>
<td>1.3 ± 0.3</td>
<td>ns</td>
</tr>
<tr>
<td>LDL-cholesterol at $t_1$, mmol/l</td>
<td>3.7 ± 0.6</td>
<td>3.0 ± 0.7</td>
<td>0.05</td>
</tr>
<tr>
<td>Triglycerides at $t_1$, mmol/l</td>
<td>1.50 ± 0.67</td>
<td>1.11 ± 0.80</td>
<td>ns</td>
</tr>
<tr>
<td>Insulin increment, IVGTT at $t_1$</td>
<td>103 ± 68</td>
<td>56 ± 42</td>
<td>ns</td>
</tr>
<tr>
<td>Insulin at $t_1$, mU/l</td>
<td>13.6 ± 6.3</td>
<td>8.9 ± 7.0</td>
<td>0.05</td>
</tr>
<tr>
<td>Glucose at $t_1$, mmol/l</td>
<td>4.8 ± 0.7</td>
<td>4.6 ± 0.6</td>
<td>ns</td>
</tr>
</tbody>
</table>
insulin concentrations in comparison with subjects in the remaining M/I quartiles, table VI. Furthermore, PCOS patients who had M/I-levels in the lowest quartile at the follow-up investigation displayed a greater weight increase over time in comparison to patients with intermediate/normal insulin sensitivity at the follow-up. However, PCOS patients in the lowest M/I quartile had similar free androgen index, total testosterone and SHBG concentrations at the index assessment in comparison with patients with intermediate/normal insulin sensitive at the follow-up investigation.

Table VII. Variables associated with M/I at the follow-up

<table>
<thead>
<tr>
<th></th>
<th>( \beta ) adjusted for age</th>
<th>( \beta )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>-0.372*</td>
<td></td>
</tr>
<tr>
<td>WHR</td>
<td>-0.502**</td>
<td>-0.463*</td>
</tr>
<tr>
<td>FAI (^b)</td>
<td>-0.100</td>
<td>-0.154</td>
</tr>
<tr>
<td>Testosterone (^b)</td>
<td>0.060</td>
<td>0.016</td>
</tr>
<tr>
<td>SHBG (^b)</td>
<td>0.353(^a)</td>
<td>0.308</td>
</tr>
<tr>
<td>CRP</td>
<td>-0.565***</td>
<td>-0.498**</td>
</tr>
<tr>
<td>ApoB/ApoA1</td>
<td>-0.509**</td>
<td>-0.460**</td>
</tr>
<tr>
<td>HDL</td>
<td>0.421*</td>
<td>0.456*</td>
</tr>
<tr>
<td>LDL</td>
<td>-0.280</td>
<td>-0.232</td>
</tr>
<tr>
<td>Total cholesterol</td>
<td>-0.162</td>
<td>-0.086</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>-0.541**</td>
<td>-0.515**</td>
</tr>
<tr>
<td>C-peptide</td>
<td>-0.425*</td>
<td>-0.324*</td>
</tr>
</tbody>
</table>

\(^a\) \( p = 0.064 \), \(^b\) three patients with combined oral contraceptives and one postmenopausal woman on hormone replacement therapy were excluded from the analysis

Variables associated with insulin sensitivity at the follow-up investigation

Table VII displays factors associated with M/I at the time of the follow-up investigation. Waist/hip ratio, hs-CRP, apoB/apoA1 ratio, HDL-cholesterol,
triglycerides, and C-peptide were all associated with insulin sensitivity even after adjustment for age. There was a trend towards a significant association with SHBG in the bivariate analysis (p = 0.064), which was lost when age was added to the model. Neither free androgen index nor total testosterone was associated with insulin sensitivity at the time of the follow-up investigation.

Paper IV

84 women with PCOS and 87 control subjects participated in paper IV. Of these, 49 performed OGTT, 32 were subjected to euglycemic hyperinsulinemic clamp/IVGTT (paper III), five subjects with known diabetes were not eligible for any of these interventions and four patients dropped out of the study prior to the clamp investigation. Six subjects participated in OGTT as well as clamp investigations. Among control subjects, 68 performed OGTT, 13 euglycemic hyperinsulinemic clamp/IVGTT (not reported in this thesis), and remaining six subjects dropped out. None of the controls participated in more than one tolerance test. Data from euglycemic hyperinsulinemic clamp/IVGTT are only used in this study for assessment of diabetes and impaired fasting glucose.

At the index assessment 36 (42.9 %) of women presented with PCO and oligomenorrhoea, 4 (4.8 %) had PCO and hyperandrogenism, and 44 (52.4 %) had PCO, oligomenorrhoea, and hyperandrogenism. At the follow-up investigation 31 (36.9 %) women still fulfilled and 30 women (35.7 %) no longer fulfilled diagnostic criteria for PCOS. In 23 women (27.4 %) PCOS criteria could not be assessed because of postmenopausal status, prior surgery or ongoing use of hormonal contraception.

Prevalence of the metabolic syndrome
According to scientific statement of AHA and NCEP/ATPIII, the prevalence of MetS in PCOS women was 21.4 % and in healthy controls 6.9 %.

Prevalence of impaired glucose tolerance and diabetes
Eighteen (21.4 %) PCOS patients had impaired glucose tolerance or diabetes at the follow-up which was significantly more common than in control subjects, p < 0.05. Two women with PCOS had developed type 1 diabetes (2.4 %), seven women had developed type 2 diabetes (8.3 %), eight women had IGT (9.5 %) and one had elevated fasting glucose (1.2 %). Four women with diabetes were currently treated with insulin. Corresponding figures for control subjects were one woman with type 2 diabetes (1.1 % diagnosed at euglycemic hyperinsulinemic clamp), two women with IGT (2.3 %), and one with elevated fasting glucose (1.1 %).
Metabolic and anthropometric parameters at the follow-up investigation

Anthropometric measures in non-diabetic women with PCOS and their healthy counterparts are displayed in table VIII, metabolic parameters in table IX, and parameters from the OGTT in table X.
Table VIII. Anthropometric measures in all non-diabetic PCOS patients, resolved and unresolved PCOS patients, PCOS phenotypic subgroups and healthy controls at the follow-up investigation.

<table>
<thead>
<tr>
<th></th>
<th>All PCOS patients&lt;sup&gt;e&lt;/sup&gt;</th>
<th>PCOS patients with persisting symptoms at follow-up&lt;sup&gt;e&lt;/sup&gt;</th>
<th>PCOS patients with resolved symptoms at follow-up&lt;sup&gt;e&lt;/sup&gt;</th>
<th>PCOS patients with hirsutism, oligomenorrhea and PCO at index assessment&lt;sup&gt;e&lt;/sup&gt;</th>
<th>PCOS patients with oligomenorrhea and PCO at index assessment&lt;sup&gt;e&lt;/sup&gt;</th>
<th>Control subjects</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>75</td>
<td>27</td>
<td>27</td>
<td>40</td>
<td>32</td>
<td>86</td>
</tr>
<tr>
<td>BMI, kg/m&lt;sup&gt;2&lt;/sup&gt;</td>
<td>27.56 ± 5.59&lt;sup&gt;a&lt;/sup&gt;</td>
<td>28.9 ± 6.0&lt;sup&gt;b,c&lt;/sup&gt;</td>
<td>25.5 ± 4.0</td>
<td>29.2 ± 5.7&lt;sup&gt;b,d&lt;/sup&gt;</td>
<td>26.2 ± 5.2</td>
<td>25.6 ± 4.2</td>
</tr>
<tr>
<td>Waist, cm</td>
<td>88.2 ± 14.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>92 ± 15&lt;sup&gt;b,c&lt;/sup&gt;</td>
<td>81 ± 11</td>
<td>91 ± 15&lt;sup&gt;b,d&lt;/sup&gt;</td>
<td>84 ± 13</td>
<td>82 ± 10</td>
</tr>
<tr>
<td>Waist:hip ratio</td>
<td>0.85 ± 0.09&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.86 ± 0.09&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.82 ± 0.08</td>
<td>0.85 ± 0.09&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.84 ± 0.08</td>
<td>0.81 ± 0.06</td>
</tr>
<tr>
<td>Fat percent, %</td>
<td>36.6 ± 9.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>39.8 ± 8.9</td>
<td>31.3 ± 8.4</td>
<td>37.8 ± 9.4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>34.6 ± 9.5</td>
<td>33.3 ± 7.4</td>
</tr>
<tr>
<td>Systolic blood pressure, mm Hg</td>
<td>132 ± 20</td>
<td>129 ± 19</td>
<td>129 ± 19</td>
<td>131 ± 20</td>
<td>133 ± 22&lt;sup&gt;b&lt;/sup&gt;</td>
<td>125 ± 16</td>
</tr>
<tr>
<td>Diastolic blood pressure, mm HG</td>
<td>82 ± 12</td>
<td>81 ± 11</td>
<td>80 ± 12</td>
<td>82 ± 13</td>
<td>83 ± 12</td>
<td>79 ± 10</td>
</tr>
</tbody>
</table>

<sup>a</sup> Significantly different from control subjects, Student’s T-test (unadjusted).

<sup>b</sup> Significantly different from control subjects, ANOVA post-hoc Tukey HSD, p < 0.05 – 0.001 (unadjusted)

<sup>c</sup> Significantly different from resolved PCOS patients, ANOVA post-hoc Tukey HSD, p < 0.05 (unadjusted)

<sup>d</sup> Significantly different from PCOS patients with oligomenorrhoea and PCO at index assessment, ANOVA post-hoc Tukey HSD, p < 0.05 – 0.001 (unadjusted)

<sup>e</sup>Women with diabetes not included
Table IX. Metabolic variables in all non-diabetic PCOS patients, resolved and unresolved PCOS patients, PCOS phenotypic subgroups and healthy controls at the follow-up investigation.

<table>
<thead>
<tr>
<th></th>
<th>All PCOS patients&lt;sup&gt;e&lt;/sup&gt;</th>
<th>PCOS patients with persisting symptoms at follow-up&lt;sup&gt;e&lt;/sup&gt;</th>
<th>PCOS patients with resolved symptoms at follow-up&lt;sup&gt;e&lt;/sup&gt;</th>
<th>PCOS patients with hirsutism, oligomenorrhea and PCO at index assessment&lt;sup&gt;f&lt;/sup&gt;</th>
<th>PCOS patients with oligomenorrhea and PCO at index assessment&lt;sup&gt;e&lt;/sup&gt;</th>
<th>Control subjects&lt;sup&gt;f&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fasting insulin, mU/l&lt;sup&gt;a&lt;/sup&gt;</td>
<td>9.78 ± 7.60</td>
<td>11.6 ± 10.2</td>
<td>8.0 ± 4.8</td>
<td>10.5 ± 8.2</td>
<td>10.9 ± 8.9</td>
<td>7.5 ± 5.8</td>
</tr>
<tr>
<td>Fasting glucose, mmol/l</td>
<td>4.71 ± 0.61</td>
<td>4.8 ± 0.6</td>
<td>4.7 ± 0.6</td>
<td>4.8 ± 0.6</td>
<td>4.7 ± 0.6</td>
<td>4.7 ± 0.5</td>
</tr>
<tr>
<td>SHBG&lt;sup&gt;b&lt;/sup&gt;</td>
<td>45.3 ± 27.1</td>
<td>36.0 ± 17.7</td>
<td>55.7 ± 27.3</td>
<td>38.0 ± 19.6</td>
<td>51.7 ± 28.0</td>
<td>60.3 ± 30.8</td>
</tr>
<tr>
<td>C-peptide, nmol/l</td>
<td>0.72 ± 0.28</td>
<td>0.75 ± 0.28</td>
<td>0.63 ± 0.20</td>
<td>0.77 ± 0.32</td>
<td>0.66 ± 0.22</td>
<td>0.62 ± 0.28</td>
</tr>
<tr>
<td>IGFBP3, mg/l</td>
<td>4.20 ± 0.86</td>
<td>4.23 ± 0.91</td>
<td>4.25 ± 0.84</td>
<td>4.09 ± 0.86</td>
<td>4.25 ± 0.85</td>
<td>4.12 ± 0.63</td>
</tr>
<tr>
<td>Hs-CRP, mg/l</td>
<td>1.91 ± 3.0</td>
<td>2.45 ± 4.16</td>
<td>0.92 ± 0.74</td>
<td>1.98 ± 2.28</td>
<td>1.95 ± 3.80</td>
<td>2.06 ± 3.36</td>
</tr>
<tr>
<td>Triglycerides, mmol/l</td>
<td>1.29 ± 0.81</td>
<td>1.28 ± 0.62</td>
<td>1.31 ± 1.02</td>
<td>1.32 ± 0.80</td>
<td>1.27 ± 0.86</td>
<td>1.01 ± 0.48</td>
</tr>
<tr>
<td>Apolipoprotein A1, g/l</td>
<td>1.47 ± 0.28</td>
<td>1.43 ± 0.30</td>
<td>1.50 ± 0.24</td>
<td>1.48 ± 0.25</td>
<td>1.47 ± 0.31</td>
<td>1.57 ± 0.28</td>
</tr>
<tr>
<td>Apolipoprotein B, g/l</td>
<td>0.91 ± 0.25</td>
<td>0.90 ± 0.24</td>
<td>0.89 ± 0.24</td>
<td>0.92 ± 0.27</td>
<td>0.90 ± 0.23</td>
<td>0.89 ± 0.26</td>
</tr>
<tr>
<td>ApoB/ApoA1 ratio</td>
<td>0.64 ± 0.22</td>
<td>0.66 ± 0.21</td>
<td>0.61 ± 0.19</td>
<td>0.64 ± 0.24</td>
<td>0.64 ± 0.21</td>
<td>0.59 ± 0.21</td>
</tr>
</tbody>
</table>

<sup>a</sup> Significantly different from control subjects, ANOVA post-hoc Tukey HSD, p < 0.05 – 0.001

<sup>b</sup> Significantly different from control subjects, Linear regression with adjustment for BMI, p 0.05 – 0.01

<sup>c</sup> Borderline difference from control subjects, Linear regression with adjustment for BMI, p = 0.054

<sup>d</sup> Significantly different from resolved PCOS patients, Linear regression with adjustment for BMI, p < 0.05

<sup>e</sup>Women with diabetes not included

<sup>f</sup>Only PCOS patients and controls without current use of combined oral contraceptives or hormone replacement therapy included in the analysis
Table X. OGTT variables in all non-diabetic PCOS patients, resolved and unresolved PCOS patients, PCOS phenotypic subgroups and healthy controls at the follow-up investigation.

<table>
<thead>
<tr>
<th></th>
<th>All PCOS patients&lt;sup&gt;e&lt;/sup&gt;</th>
<th>PCOS patients with persisting symptoms at follow-up&lt;sup&gt;e&lt;/sup&gt;</th>
<th>PCOS patients with resolved symptoms at follow-up&lt;sup&gt;e&lt;/sup&gt;</th>
<th>PCOS patients with hirsutism, oligomenorrhea and PCO at index assessment&lt;sup&gt;e&lt;/sup&gt;</th>
<th>PCOS patients with oligomenorrhea and PCO at index assessment&lt;sup&gt;e&lt;/sup&gt;</th>
<th>Control subjects</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n = 46</td>
<td>n = 19</td>
<td>n = 18</td>
<td>n = 20</td>
<td>n = 24</td>
<td>n = 68</td>
</tr>
<tr>
<td>30-minute glucose, OGTT, mmol/l</td>
<td>7.6 ± 2.5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>7.8 ± 3.0&lt;sup&gt;b&lt;/sup&gt;</td>
<td>7.5 ± 2.0</td>
<td>8.2 ± 2.6&lt;sup&gt;b&lt;/sup&gt;</td>
<td>7.3 ± 2.3</td>
<td>6.4 ± 1.3</td>
</tr>
<tr>
<td>120-minute glucose, OGTT, mmol/l</td>
<td>5.3 ± 1.9</td>
<td>5.6 ± 2.1</td>
<td>4.9 ± 1.8</td>
<td>5.7 ± 1.8</td>
<td>5.0 ± 2.0</td>
<td>4.7 ± 1.3</td>
</tr>
<tr>
<td>30-minute insulin, OGTT, mU/l</td>
<td>81.7 ± 63.8&lt;sup&gt;b&lt;/sup&gt;</td>
<td>87.3 ± 66.0&lt;sup&gt;b&lt;/sup&gt;</td>
<td>78.6 ± 67.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>97.0 ± 67.4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>72.2 ± 61.4</td>
<td>42.3 ± 20.9</td>
</tr>
<tr>
<td>120-minute insulin, OGTT, mU/l</td>
<td>72.7 ± 87.8&lt;sup&gt;b&lt;/sup&gt;</td>
<td>72.4 ± 95.2</td>
<td>57.8 ± 50.7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>88.5 ± 106.6&lt;sup&gt;b&lt;/sup&gt;</td>
<td>61.5 ± 72.7</td>
<td>27.4 ± 25.1</td>
</tr>
<tr>
<td>Matsuda Index</td>
<td>2.01 ± 3.63&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.23 ± 4.45&lt;sup&gt;c&lt;/sup&gt;</td>
<td>2.16 ± 3.53&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.64 ± 3.99&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.36 ± 3.51&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.43 ± 3.99</td>
</tr>
<tr>
<td>Insulinogenic index</td>
<td>8.93 ± 6.35&lt;sup&gt;b&lt;/sup&gt;</td>
<td>9.72 ± 7.51&lt;sup&gt;c&lt;/sup&gt;</td>
<td>8.64 ± 6.04&lt;sup&gt;a&lt;/sup&gt;</td>
<td>10.21 ± 7.28&lt;sup&gt;b&lt;/sup&gt;</td>
<td>8.10 ± 5.67&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.50 ± 2.97</td>
</tr>
</tbody>
</table>

<sup>a</sup> Significantly different from control subjects, ANOVA post-hoc Tukey HSD, p < 0.05 – 0.001
<sup>b</sup> Significantly different from control subjects, Linear regression with adjustment for BMI, p < 0.05 - 0.001
<sup>c</sup> Borderline differences from control subjects, Linear regression with adjustment for BMI, p = 0.054 and p = 0.078
<sup>d</sup> Significantly different from resolved PCOS patients, Linear regression with adjustment for BMI, p < 0.05
<sup>e</sup> Women with diabetes not included
PCOS patients had higher BMI, increased waist, increased waist/hip ratio, and increased fat percent in comparison with control subjects why subsequent statistical analyses have been adjusted for BMI, table VIII. Following the adjustment for BMI, insulin sensitivity measured by Matsuda insulin sensitivity index was significantly lower in women with PCOS, table X. The insulinogenic index as a measure of beta-cell function was elevated in PCOS patients, table X. In addition, women with PCOS had elevated levels of proinsulin, even after adjustment for BMI, table IX.

Metabolic and anthropometric parameters depending on PCOS phenotype at the initial assessment

PCOS patients with clinical signs of hyperandrogenism in addition to menstrual disorders and PCO at index assessment had increased BMI, waist circumference, waist-hip ratio, and fat percent than controls, table VIII. BMI and waist circumference were even higher than in PCOS patients with menstrual irregularities and PCO only at index assessment. Otherwise there were no differences between women with different phenotypic expressions at the index assessment in lipid profile, inflammatory markers or parameters of insulin metabolism at follow-up investigation.

Women without clinical signs of hyperandrogenism at the index assessment displayed higher fasting insulin and proinsulin plasma concentrations than controls. In addition, they had lower Matsuda insulin sensitivity index and higher insulinogenic index than controls, table IX and X.

When adjusted for BMI, there was also a trend towards significantly lower insulin sensitivity and increased insulinogenic index among women with hyperandrogenism at the index assessment in comparison with control subjects. Women with hyperandrogenic PCOS phenotype at the index assessment had lower serum concentrations of SHBG than controls at the follow-up investigation.

Metabolic and anthropometric parameters depending on persisting PCOS symptoms

Women with persisting PCOS symptoms had increased waist circumference, higher WHR and BMI than controls, whereas women with resolved PCOS did not differ in any of these aspects from control subjects, table VIII. BMI and waist circumference in women with persisting PCOS symptoms were also increased in comparison to women with resolved PCOS symptoms at the follow-up investigation, table VIII and IX. With the exception of lower SHBG levels among women with unresolved PCOS, there were no differences between women with resolved and unresolved PCOS in lipid profile, inflammatory markers or parameters of insulin metabolism.
Table XI. Results of a multivariate linear regression analysis of variables associated with insulin sensitivity expressed by Matsuda Index

<table>
<thead>
<tr>
<th>Variable</th>
<th>B</th>
<th>95 % CI</th>
<th>β</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group</td>
<td>-0.183</td>
<td>-0.354 - -0.011</td>
<td>-0.144</td>
<td>0.05</td>
</tr>
<tr>
<td>Age</td>
<td>-0.019</td>
<td>-0.034 - -0.004</td>
<td>-0.159</td>
<td>0.05</td>
</tr>
<tr>
<td>BMI</td>
<td>-0.055</td>
<td>-0.076 - -0.034</td>
<td>-0.401</td>
<td>0.001</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>-0.242</td>
<td>-0.368 - -0.115</td>
<td>-0.272</td>
<td>0.001</td>
</tr>
<tr>
<td>SHBG</td>
<td>0.003</td>
<td>0.001 – 0.006</td>
<td>0.175</td>
<td>0.05</td>
</tr>
<tr>
<td>hs-CRP</td>
<td>-0.023</td>
<td>-0.54 – 0.008</td>
<td>-0.102</td>
<td>ns</td>
</tr>
</tbody>
</table>

Variables entered into the equation are based on significant findings in bivariate analyses and colinearity analyses. R² for final model was 0.62.

Insulin sensitivity measured as Matsuda index differed from controls in both women with persisting and resolved PCOS at the follow-up investigation. Likewise, beta cell function measured as insulinogenic index was significantly elevated in both women with resolved and unresolved PCOS in comparison with control subjects, table X.

*Variables associated with insulin sensitivity at the follow-up investigation*

Table XI displays factors associated with Matsuda insulin sensitivity index at the time of the follow-up investigation. Age, BMI, triglycerides and low SHBG levels, together with PCOS diagnosis (resolved as well as unresolved) were all significantly and independently associated with insulin sensitivity. Free androgen index was not associated with insulin sensitivity in the bivariate analysis, and was hence not brought into the final model.
Discussion

Methodological considerations

The emphasis in this study has been to provide the twenty-year old, newly diagnosed PCOS patient with relevant information as regards her future reproductive health and her future risk of developing cardiovascular and metabolic diseases. From her perspective, there is a fair chance that her PCOS will resolve with advancing age. In order to provide useful information to clinicians who meet young, internet-surfing women with PCOS we needed to include all our patients in the long-term analyses, not only the ones who continued to meet PCOS criteria in their forties. If only women with persisting PCOS had been included, we would have investigated a selected subgroup with worse outcome and the young patients would be presented with exaggerated risk figures.

Like any follow-up study, free living subjects tend to develop medical disorders and utilize medications which may have confounded our results. Besides the use of antihypertensive drugs which may affect the pulse wave analysis, our patients were also using various types of contraception. In addition we also had a variable endocrine status among our PCOS patients with some menopausal subjects and with premenopausal subjects evaluated in the follicular or luteal phases of the menstrual cycle. If we had limited our study to patients with similar endocrine status we would have ended up with a much more limited sample for the follow-up investigation. Furthermore, many of our participating patients had to travel to Uppsala for 2 – 5 hours and a few also stayed over night. Given the efforts patients went through to participate in the study, we felt that it would be too much to ask to schedule their appointments according to menstrual cycle phase. Also, it was not feasible to request from patients that they should discontinue their use of oral contraceptives/HRT prior to participation.

As we knew beforehand that variable endocrine status was to be expected we tried to use outcome measures that were not affected by menstrual cycle phase. For instance, endothelial function was evaluated by pulse wave analysis instead of flow-mediated dilatation of the brachial artery where changes across the menstrual cycle have been reported. However, for many
of the outcome measures in paper I this was not feasible, why data are reported in subgroups with more uniform endocrine status.

The choice of the control group was a major concern when this study was planned. In the end, and with the young PCOS patient in perspective, we decided upon using a population-based control group. The use of a weight-matched control group was thoroughly discussed but discarded for several reasons. First, with the hypothesis (based on our clinical experience) that a proportion of PCOS patients improve with increasing age, and as it can be assumed that a weight-matched control group also would present with diabetes and hypertension at this age, we would risk underestimating the burden of PCOS with the use of a weight-matched control group. Secondly, by using a population-based age-matched control group, we would obtain a more reliable overall picture of PCOS patients’ risk profile in comparison with women of similar age. Third, we also had the possibility to adjust all statistical analyses for BMI. Finally, finding a weight-matched population without using selected hospital-based populations was not considered possible.

Another problem with the control group was that women with PCOS were identified in the past, whereas control subjects were identified in the present. Given the resolution of PCOS in many of the cases, it is thus possible that there is a contamination in the control population. When the study was planned, we did discuss the possibility of using patient groups with infertility causes other than PCOS who had presented at the IVF clinic between 1987 and 1995 as controls. Again, this suggestion was rejected for ethical reasons and because life-time reproductive outcome also was one of our aims.

The precautions undertaken to ensure that no PCOS cases were hidden among controls were 1) transvaginal ultrasound screening (although it could be assumed that some controls would have had PCO if investigated at an earlier age) and 2) all controls were screened for history of oligomenorrhea or amenorrhea. In fact, the question we asked was “Have you ever, on more than one occasion, had more than six weeks between menses without being pregnant?” A similar question was asked for amenorrhea. One control subject reported a history of amenorrhea (3-4 months) during her college-year in the US, but as that was more likely due to stress she was kept in the study. None of our controls had polycystic ovaries on ultrasound.

In addition, the invitation letter to control subjects specifically described that they would act as a control group to women with PCOS. We also described the symptoms and problems of PCOS in the letter so that potential control subjects could judge for themselves whether or not they were
suitable for the study. Upon initial contact two potential control subjects were excluded because they identified themselves as PCOS patients.

Reproductive outcome

Reproductive outcome did not differ between this unselected population of women with a previous diagnosis of PCOS and healthy control subjects, and the ovarian reserve seemed better preserved in the women with PCOS. The live birth rate and the rate of miscarriages were similar in PCOS patients and control women.

In our study almost all PCOS patients who attempted a pregnancy were successful. The cumulative delivery rate in our subjects was almost 90 %, which is in line with a population-based study of PCOS patients [151] and a previous long-term follow-up study of infertile women with ovulatory disorders, where a cumulative delivery rate of 82 % was reported [152]. More than two thirds of the PCOS patients in the present study reported at least one spontaneous pregnancy.

The menstrual frequency among PCOS patients who were premenopausal and without hormonal contraception was lower than in control subjects, but PCOS patients reported on average almost 9 menstrual cycles per year. Some clinical observations suggest improved fertility in ageing women with PCOS. A positive impact of ageing on cycle regularization in PCOS has recently been claimed, but the fertility outcome was not evaluated [138]. The authors concluded that the follicle loss through the process of ovarian ageing could explain the occurrence of regular cycles in older patients with PCOS [138]. In women overall, ageing is associated with a subtle shortening of menstrual cycles of a mean of 2-3 days from the age 20 to the early forties [49, 153]. Most likely, the mechanism is associated with a reduction in antral follicle numbers, and consequently a more rapid follicular growth and selection, thus shortening of the follicular phase. Careful monitoring has suggested that basal FSH levels are lower in young women with PCOS than in the early follicular phase of women with normal ovaries [59]. The mechanism behind these low levels, which may partly explain lack of follicular growth, is probably increased production of inhibin B from the increased number of antral follicles in polycystic ovaries [154]. Ovarian ageing results in diminution of the follicular cohort in both normal women and PCOS patients, and is associated with decreased inhibin B and AMH levels [48, 50]. Ageing may thus be followed by lower inhibin B levels, which will permit FSH enhancement and lead to full follicle maturation, more regular menstrual cycles and the appearance of ovulatory cycles in polycystic ovaries. A similar mechanism may be responsible for the increased number of ovulatory cycles after ovarian wedge resection or ovarian drilling, when the cohort of resting follicles is dramatically reduced [155]. Thus, in women with PCOS, such an age-dependent reduction in the
pool of resting follicles may eventually lead to more cycles becoming ovulatory and a catch-up of fecundity.

The rate of miscarriages was not increased in PCOS patients and the number of subjects who had had at least one miscarriage throughout their fertile life did not differ in comparison with the control subjects. These findings are at odds with previous reports of increased rates of miscarriages in PCOS patients [41-43]. The discrepancy is most likely due to the fact that previous studies have merely included infertile PCOS patients undergoing assisted reproduction, whereas the present study included an unselected population, i.e. PCOS patients seeking help for various causes such as oligomenorrhea, hirsutism or infertility. However, more than half of our PCOS patients had at some point been treated for infertility. Our findings suggest that other factors than the PCOS diagnosis might contribute to the increased risk of miscarriage reported in prior studies, such as obesity, fertility treatment [41] or smoking [156]. Indeed, in women with polycystic ovaries, but not a full syndrome, miscarriage rates have been reported to be normal [151], as has prevalence of recurrent miscarriages [44].

Ovarian reserve
Mean ovarian volume and number of antral follicles, measures of ovarian reserve, were significantly higher in PCOS patients. Likewise, PCOS patients had markedly higher serum concentrations of AMH. A number of previous reports for young women with PCOS lend support for an increased ovarian reserve in these women compared with age-matched controls [50]. Thus, polycystic ovaries are larger and contain more antral follicles as assessed by ovarian ultrasound [66, 67], and density of follicles at primary stages is increased [34]. Several studies have also reported higher levels of AMH in women with PCOS than in controls [35, 56, 57]. Findings on inhibin B are more discrepant [64, 65]. Our findings in older women with PCOS are in accordance with these results, suggesting a retained greater ovarian reserve in these women in their forties. Results published for basal levels of FSH in PCOS have been less conclusive [58]. However, Dahlgren and colleagues found lower levels of FSH in 50-year old women with a previous PCOS diagnosis compared with controls, in spite of the fact that the women with PCOS had been wedge-resected, which is in line with our findings. Recently, it was proposed that serum FSH assessments are inferior to measurements of AMH in identifying women with reduced ovarian reserve, largely because AMH is cycle-independent [51]. This assumption is substantiated in the present study, where AMH levels correlated strongly both with ovarian volume and follicle counts. Recent results indicate that AMH is a strong predictor for success rates of assisted reproduction, i.e. ovarian reserve [56, 157, 158], underlining the importance of the vast
differences in AMH levels we found between women with PCOS and controls.

Also, the menstrual cycle length has been reported to be an age-independent marker of female fertility. Recently, it was shown that the mean menstrual cycle length correlated linearly with pregnancy and delivery rates after IVF/ICSI, even after age adjustment [49]. Although women with anovulatory PCOS were not included in that study, the authors found an association between mean menstrual cycle length and antral follicle counts, many of the patients with cycles > 28 days being women with multifollicular or ovulating polycystic ovaries. In line with those findings, others [159, 160] reported on higher pregnancy rates after IVF in women with PCOS than in women with normal ovaries.

Endothelial dysfunction

PCOS patients, who at the time of follow-up had reached a mean age of 43 years, displayed signs of deteriorated endothelium-dependent vasodilatation in comparison to age-matched healthy controls and this finding was mainly driven by patients who still fulfilled diagnostic criteria for PCOS. However, this difference was primarily explained by the increased prevalence of cardiovascular risk factors among the PCOS patients, such as increased BMI and co-morbid diabetes, hypertension, and hyperlipidemia. Indeed, when the statistical analyses on endothelial function were adjusted for one of these risk factors, BMI, none of the significant differences between groups remained. This finding is in line with previous studies from our group, suggesting that PCOS per se is not associated with any metabolic disturbances that can be distinguished from co-morbid obesity [23, 85].

Endothelial function is influenced by a number of factors. Previous studies have linked obesity to endothelial dysfunction [161-166], and recently Lind and colleagues demonstrated that EDV in resistance arteries was reduced in subjects with increased intra-abdominal adipose tissue mass [167]. EDV is also associated with insulin resistance and reduced in subjects with the metabolic syndrome [149]. In our middle-aged PCOS patients insulin resistance was a stronger independent explanatory variable of EDV than BMI. This finding is supported by studies of younger PCOS patients [132, 135] and by the fact that treatments aiming at decreasing insulin resistance in PCOS patients, such as metformin, have been shown to improve endothelial function [168-171].

A higher prevalence of cardiovascular risk factors in patients with PCOS has been described in multiple studies [124-128]. In the line with these studies we have confirmed that PCOS patients between 40 – 50 years of age
present with increased BMI, waist/hip ratio, systolic and diastolic blood pressures and triglycerides. Presumably because of the relatively low age of subjects, we failed to demonstrate a higher prevalence of cardiovascular events in patients with PCOS.

**Metabolic syndrome**

According to the scientific statement of AHA and NCEP/ATPIII, the prevalence of MetS in PCOS women was 21.4 % and in healthy controls 6.9 % in our study. This was nearly 2-fold lower than in previously published American and Asian studies [68, 107, 108, 172, 173], presumably because of lower BMI and differences in racial/ethnic composition of our probands in comparison to these studies.

In addition, frequency of isolated features of metabolic syndrome was more common in women with PCOS. This is in line with studies from central Europe and China who reported relatively low prevalence of overt metabolic syndrome, but frequent occurrence of metabolic and anthropometric disturbances [105, 109].

PCOS women between 40 – 50 years of age presented in our study with increased waist circumference and waist/hip ratio, BMI and triglycerides. These metabolic and anthropometric parameters and even HDL were also associated with insulin sensitivity. Both systolic and diastolic blood pressures were higher in PCOS women. This possibly pre-hypertensive state with increased day-time blood pressures in women with PCOS, persisting even after adjusting for BMI, body fat distribution and insulin resistance, was previously described in this group of women already at the index assessment [112]. Surprisingly, the regular intake of antihypertensive drugs at the follow-up was not higher in PCOS women than in controls. Further analyses are mandatory to reveal whether BMI and age are stronger contributing factors than hyperandrogenism and insulin resistance [109]. This hypothesis is supported by our results in Paper III, where insulin sensitivity ratio M/I was lower in women with high BMI at the index assessment and in those who progressively gained weight until the follow-up.

**Hyperandrogenism**

PCOS patients displayed decreasing testosterone levels over time but they still had increased free androgen index compared to controls at the time of the follow-up. Clearly, although their menstrual frequency might be normalized across time, the androgen levels remain increased in comparison to controls. Persistently elevated androgen levels have previously been demonstrated in older PCOS patients in comparison with age-matched controls [174].
The decreasing testosterone levels over time is in line with a cross-sectional study in PCOS patients suggesting 40–60% lower levels of total testosterone in PCOS patients 42 years or older as compared to younger PCOS patients [174]. In contrast to PCOS patients, a longitudinal study in healthy women recently showed that testosterone levels display a gradual increase from the age of 40 and onwards [175].

It has been argued that the combination of hyperandrogenism and menstrual irregularity is associated with the most profound insulin resistance [176] and some studies even indicate that PCOS patients without clinical features of hyperandrogenism do not develop insulin resistance [88, 89]. According to our findings the phenotypic presentation at the index assessment had no influence on insulin sensitivity at the time of the follow-up. Both women with oligomenorrhea and PCOS as well as women with hyperandrogenism, oligomenorrhea and PCO displayed decreased insulin sensitivity, expressed as Matsuda Index, in comparison with control subjects and there was no difference in insulin sensitivity between PCOS phenotypic expressions. However, hyperandrogenism is less relevant for insulin sensitivity when women with PCOS have reached premenopausal ages, which is further corroborated by the fact that free androgen index is no longer associated with Matsuda index in the bivariate linear regression. Insulin sensitivity among our premenopausal women was instead independently associated with age, BMI, triglycerides, SHBG and PCOS diagnosis (resolved or unresolved). In the long run, however, women who presented with hyperandrogenism at the index presentation may presumably suffer an increased risk of subsequent development of type 2 diabetes as they had increased BMI, increased waist circumference and reduced SHBG levels in comparison with control subjects, all of these factors individually contributing to their life-time risk of diabetes [101-103, 177-180].

Previous studies have firmly concluded that SHBG is associated with insulin sensitivity in both women with PCOS and other women [178, 179]. Among our PCOS women in paper III we found a trend towards a significant association between SHBG and insulin sensitivity, but only in the bivariate analysis. In paper IV, on the contrary, SHBG was one of the factors independently associated with insulin sensitivity (see also chapter Insulin resistance, glucose tolerance, early insulin response and diabetes mellitus). The absence of a stronger relationship in paper III is presumably due to the relatively small sample size or the relatively high proportion of lean subjects.

SHBG increased over time which is at odds with findings in healthy women. Longitudinal studies in non-PCOS women indicate that increased BMI over time is closely related to lowering of SHBG levels [181]. However, decreasing SHBG levels over time has also been reported to occur.
in women irrespective of BMI changes, thus increasing the biologically available testosterone in pre- and perimenopausal women [175]. The changes in testosterone and SHBG levels over time in our cohort of women with PCOS suggest that hyperandrogenism may partly resolve before menopause in this group of women. This finding is in line with previous findings of an improved menstrual cycle pattern with increasing age in PCOS patients [61, 182] and the fact that in at least one third of our women no longer fulfilled PCOS criteria.

**Insulin resistance, glucose tolerance, early insulin response and diabetes mellitus**

Prevalence of disturbances of glucose metabolism was significantly higher in PCOS women in comparison with healthy controls. Only 4.5 % of controls had impaired glucose tolerance or type 2 diabetes while in women with PCOS the corresponding figure was 16.7 % (if also cases with type 1 diabetes are included the corresponding figure is 21.4 %). This figure is, however, substantially lower than previously presented [101, 102]. Prevalence rates for glucose intolerance are reported to vary between 38 - 45 % in younger women with PCOS but these studies have included subjects who predominantly are overweight or obese. However, our data fits better with previous findings in lean women with PCOS where approximately 12 % are reported to display glucose intolerance (IGT or type 2 diabetes), in particular taking into consideration that our subjects had reached a more advanced age than subjects of previous studies. The overall relatively low rate of IGT and diabetes in our population are most likely explained by lower BMI and differences in racial/ethnic composition of our probands in comparison to previous studies. Possibly, also the inclusion of milder PCOS phenotypes may have contributed to the overall lower prevalence rates for IGT and type 2 diabetes, even if hyperandrogenism seems to be less relevant for insulin sensitivity in women with PCOS in their premenopausal age (see previous chapter Hyperandrogenism).

Insulin resistance and decreased insulin secretion, and the interaction between low acute insulin response and high insulin resistance are important predisposing factors in development of type 2 diabetes [183-185]. In our study, PCOS patients over time displayed decreased acute insulin response in the IVGTT and unchanged insulin sensitivity. The former finding might reflect impaired beta-cell function with increasing age, also suggested by increased AUC glucose during the IVGTT. Furthermore, this finding might confirm the theory of Cho and colleagues that the progression from PCOS to the development of type 2 diabetes is rather due to a progressive
deterioration of beta-cell function than further increase in insulin resistance [186]. Whether the deterioration of beta-cell function is genetically determined or reflects their exhaustion after compensation of insulin resistance over long time or even both, remain unclear [71, 187, 188]. Possible reasons why insulin sensitivity remained unchanged in our cohort may be due to a substantial fraction of women with resolved PCOS by unchanged WHR across time, or by decreasing androgen levels across time.

Another important finding of our study is that insulin sensitivity was decreased compared with controls independent of whether women had persisting or resolved PCOS symptoms at the time of the follow-up. This finding indicates that although the clinical presentation of PCOS may be less obvious when women reach the age of 40 [61, 182], their risk of developing type 2 diabetes may not have diminished. This finding also emphasizes the need to monitor women with PCOS with respect to glucose tolerance, regardless of whether they are symptomatic or symptom free.

The insulinogenic index during OGTT, as a measure of beta cell function, was increased in all PCOS patients, independent of whether PCOS symptoms were resolved or unresolved and also independent of the phenotypic presentation at the index assessment. The insulinogenic index has previously been shown to strongly correlate with the first phase insulin response following IVGTT [189] and prior findings of our group have indicated that PCOS women, independent of body weight, display an enhanced early insulin response during IVGTT [23, 85, 190]. Notably, whereas insulin sensitivity is restored following weight reduction, early insulin response is not reversed after weight reduction [23]. Whereas prior studies in young PCOS patients indicate that the increased early insulin response was not accounted for by the insulin resistance [85], the increased beta cell function of our premenopausal women with PCOS appears to be a normal beta cell adaptation to the decreased insulin sensitivity.

Insulin sensitivity also remained unchanged between the two time-points of assessment. However, PCOS patients who already at young age presented with obesity and continued to gain weight were more prone to be insulin resistant at follow-up. Function of pancreatic beta cells decreases over time in PCOS patients, presumably both due to aging and exhaustion of already genetically altered pancreatic cells compensating insulin resistance in the long term.
General conclusions

Most women with PCOS had given birth and the rate of spontaneous pregnancies was relatively high. The rate of miscarriages was not increased in PCOS patients and the number of subjects who had had at least one miscarriage throughout their fertile life did not differ in comparison with the control subjects.

The ultrasound findings together with increased levels of AMH imply that PCOS patients have an ovarian reserve possibly superior to women with normal ovaries.

PCOS women display signs of endothelial dysfunction at follow-up, but this is largely due to the increased prevalence of independent risk factors for CVD found in this group. This finding might provide support to the hypothesis that PCOS patients have a cluster of factors that increase risk of CVD but that the syndrome per se is not the predictor of cardiovascular events.

Free androgen levels decrease over time in PCOS patients but are still elevated compared to age-matched control subjects.

Insulin sensitivity remained unchanged between the two time-points of assessment but premenopausal women with PCOS had lower insulin sensitivity than age-matched controls.

PCOS patients who already at young age presented with obesity and continued to gain weight were more prone to be insulin resistant at follow-up.

Function of pancreatic beta cells decreases over time in PCOS patients, but premenopausal women with PCOS displayed increased beta cell function in comparison with age-matched control subjects.

IGT and type 2 diabetes occurred more often in PCOS patients than in healthy controls.
Independent of PCOS phenotype at the index assessment and persistence of PCOS symptoms at the follow-up investigation, premenopausal women with PCOS had lower insulin sensitivity and increased beta cell function in comparison with control subjects.
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