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Polycystic Ovary Syndrome and Pregnancy

Prenatal Exposures and Pregnancy Complications

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Abstract

Valgeirsdóttir, H. 2021. Polycystic Ovary Syndrome and Pregnancy. Prenatal Exposures and Pregnancy Complications. *Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine* 1723. 71 pp. Uppsala: Acta Universitatis Upsaliensis. ISBN 978-91-513-1153-1.

Polycystic ovary syndrome (PCOS) is the most common endocrine disorder in women of fertile age. The aetiology of PCOS is not fully understood and might be affected by foetal exposures. Women with PCOS have an increased risk of pregnancy complications, but information on rare severe complications is scarce.

The overall aim of this thesis was to gain further knowledge of the association between the intrauterine environment and development of PCOS in offspring, and the association between maternal PCOS and adverse pregnancy outcomes, with a focus on preterm birth and stillbirth.

This thesis includes three nationwide register-based cohort studies and one matched cohort study including early second-trimester blood samples. Associations were estimated with multivariate Cox, logistic and linear regression models, with adjustment for confounders including body mass index. Correlations were estimated with Spearman's rank correlation coefficient.

It was found that maternal overweight and obesity, and smoking during pregnancy, were associated with increased risk for female offspring to develop PCOS later in life compared with offspring of normal weight and non-smoking mothers, respectively. Size at birth was not associated with the risk of PCOS development. During pregnancy, women with PCOS had higher second-trimester levels of anti-Müllerian hormone (AMH) and testosterone than non-PCOS women, and AMH levels were positively correlated with total testosterone levels. High AMH levels were not associated with an increased risk of pregnancy complications. Women with PCOS seemed at increased risk of extremely, very, and moderately preterm birth compared with non-PCOS women. The association was strongest for extremely preterm birth of spontaneous onset. Women with PCOS also seemed at increased risk of stillbirth compared with non-PCOS women, and the rate of stillbirth in PCOS women was particularly high in term pregnancy.

In conclusion, increased maternal BMI and maternal smoking may increase the risk of PCOS in offspring. Even though second-trimester AMH levels are higher in pregnant women with PCOS than controls, AMH seems not to be a mediator of increased risk for pregnancy complications in PCOS women. PCOS should be considered a risk factor for severe pregnancy complications such as extremely preterm birth and stillbirth.

Keywords: Polycystic ovary syndrome, PCOS, pregnancy, epidemiology, prenatal exposures, pregnancy complications, Anti-Müllerian hormone, AMH, preterm birth, stillbirth

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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.

I **Valgeirsdottir H**, Vanky E, Sundström-Poromaa I, Roos N, Løvvik TS, Stephansson O, Wikström AK. Prenatal exposures and birth indices, and subsequent risk of polycystic ovary syndrome: a national registry-based cohort study. *BJOG*, 2019; 126(2):244–51.

II Valdimarsdottir R*, **Valgeirsdottir H***, Wikström AK, Kallak TK, Elenis E, Axelsson O, Ubhayasekhera K, Bergquist J, Piltonen TT, Pigny P, Giacopini P, Poromaa IS. Pregnancy and neonatal complications in women with polycystic ovary syndrome in relation to second-trimester anti-Müllerian hormone levels. *Reproductive Biomedicine Online*, 2019; 39(1):141–8.

III **Valgeirsdottir H**, Sundstrom Poromaa I, Kunovac Kallak T, Vanky E, Akhter T, Roos N, Stephansson O, Wikström AK. Polycystic ovary syndrome and extremely preterm birth: a nationwide register-based study. *PloS One,* 2021; 16(2):e0246743.

IV **Valgeirsdottir H**, Kallak TK, Sundström-Poromaa I, Jonsson M, Stephansson O, Roos N, Lindström L, Wikström AK. Polycystic ovary syndrome and risk of stillbirth: a nationwide register-based study. *Manuscript.*

*Both authors contributed equally to this work.

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Contents

Abbreviations

AE-PCOS	Androgen Excess and PCOS Society
AGA	Appropriate for gestational age
aHR	Adjusted hazard ratio
AMH	Anti-Müllerian hormone
aOR	Adjusted odds ratio
ART	Assisted reproductive technology
BMI	Body mass index
CI	Confidence interval
DAG	Directed acyclic graph
FAI	Free androgen index
GWG	Gestational weight gain
HR	Hazard ratio
ICD	International classification of diseases
LGA	Large for gestational age
MBR	Swedish medical birth register
OR	Odds ratio
PAF	Population attributable fraction
PCOS	Polycystic ovary syndrome
PPROM	Preterm prelabour rupture of membranes
SGA	Small for gestational age
SHBG	Sex hormone-binding globulin
WHO	World Health Organization

Introduction

Definition of PCOS

Polycystic ovary syndrome (PCOS, previously also called Stein-Leventhal syndrome) is an endocrine condition affecting 6–10% of women.[1] In addition to polycystic ovarian morphology, the syndrome is associated with hyperandrogenism and menstrual irregularity suggestive of oligo- or anovulation.[2] The criteria for PCOS have changed over time and there is still no worldwide consensus on which criteria should be used. Until 1990, the criterion for Stein-Leventhal syndrome – consisting of enlarged ovaries together with anovulation[3] – was used for the diagnosis of PCOS in Sweden. The National Institutes of Health PCOS diagnosis criteria were introduced in 1990. According to these criteria, a woman needs to have both clinical or biochemical hyperandrogenism and chronic anovulation.[4] In 2003, the Rotterdam criteria were swiftly introduced in clinical practice in Sweden. Under them, a woman needs to fulfil two out of three criteria for PCOS diagnosis: oligo- or anovulation, clinical or biochemical hyperandrogenism, and polycystic morphology of the ovaries shown with ultrasound imaging.[2] Not long after, the Androgen Excess and PCOS Society (AE-PCOS) in 2009 put forward their own PCOS criteria, emphasising hyperandrogenism to a greater extent than the Rotterdam criteria. The AE-PCOS criteria calls for the presence of both hyperandrogenism and ovarian dysfunction (oligo-anovulation and/or polycystic ovaries).[5] All criteria in use over the past 30 years have underlined the importance of excluding other endocrine disorders before PCOS is diagnosed.

Oligo-anovulation

Ovulation pattern in women can most easily be evaluated through their menstrual cycles, where amenorrhea may reflect anovulation and oligomenorrhea reflects oligo- or anovulation. Primary amenorrhea is defined as no sign of menarche at age of 15 or no menarche within 3 years from the beginning of breast development.[6] Secondary amenorrhea refers to no menstruation for 3–6 months in already menstruating women.[7, 8] Oligomenorrhea is defined as less than nine menstrual cycles a year.[7]

Polycystic ovarian morphology

Ovarian morphology is assessed through transvaginal ultrasound. The criteria for polycystic ovarian morphology were originally the presence of at least 12 follicles measuring 2–9 mm (antral follicles) in the ovary, or ovarian size over 10 ml.[2] Since ultrasonic devices have developed greatly in the last decade and ultrasound quality has improved, many more recent recommendations give 20 antral follicles as the cut-off for polycystic ovarian morphology (The Centre for Research Excellence in Polycystic Ovary Syndrome, the European Society of Human Reproduction and Embryology and American Society of Reproductive medicine),[9] some even going as high as 25 (AE-PCOS).[5] International guidelines from 2018 have also emphasised that ovarian morphology should not be assessed in adolescents, as antral follicle counts are high during and a few years after puberty.[10]

Hyperandrogenism

In women, androgens are mainly produced by the ovaries and the adrenal glands. Testosterone is probably also produced in adipose tissue, according to a recent study on rats.[11] Testosterone is synthesised from cholesterol via a chain of enzymes including 17α-hydroxylase, 3β-hydroxysteroid dehydrogenase and 17-hydroxysteroid dehydrogenase. About 50% of circulating testosterone is derived from androstenedione, converted in the liver and adipose tissue.[12] The most potent androgens are testosterone and dihydrotestosterone, whereas less potent androgens include dehydroepiandrosterone sulfate, dehydroepiandrosterone and androstenedione.[13] In the circulation, testosterone mainly occurs bound to sex hormone-binding globulin (SHBG), corticosteroid-binding globulin or albumin, and only a small percentage is free and biologically active.[14] Women with hyperandrogenism have higher circulating levels of free testosterone. Biologically active testosterone, also known as the free androgen index (FAI), is calculated as the ratio between total testosterone and SHBG. A FAI value of 0.05 is often used as a cut-off for hyperandrogenism.[15] Hirsutism, acne, and alopecia are clinical symptoms of hyperandrogenism.

Aetiology of PCOS

The aetiology of PCOS is multifactorial and not fully understood. Evidence suggests that genetic and environmental factors together play a role in the pathogenesis of PCOS.[4, 16, 17] Several genes have been identified to associate with PCOS[18-20] and diverse genes seem responsible for different phenotypes of PCOS,[21] implying a complex genetic disorder. One study has also indicated that women whose mothers had diabetes had higher risk of being diagnosed

with PCOS.[16] Twin studies have manifested higher correlations of PCOS in monozygotic than dizygotic female twins,[22, 23] manifesting the genetic component of PCOS.

Whether the prevalence of PCOS differs throughout the world and is different between different racial and ethnic groups is debatable. Conclusions on this are hard to draw, since studies on PCOS prevalence in specific groups are heterogenic in design, as regards both which diagnostic criteria are used and how the study populations are selected.[24] Still, lifestyle seems to influence the risk of developing PCOS.[25] Obesity and weight gain are associated with PCOS status,[26] whereas weight loss and increased physical activity may ameliorate PCOS symptoms.[10] Smoking increases fasting insulin levels and FAI in women with PCOS,[27] resulting in increased hyperandrogenism. Insulin resistance and hyperinsulinemia are also overrepresented in PCOS women. Insulin stimulates androgen production in the ovaries and inhibits SHBG synthesis, ultimately leading to higher concentrations of free androgens and a vicious circle for the affected women,[28] see Figure 1.

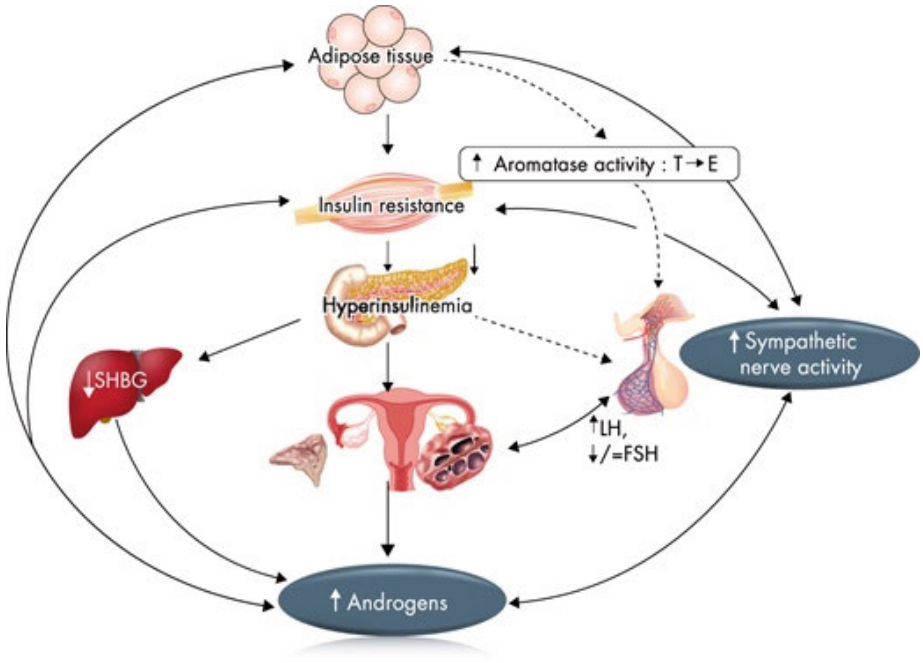

Figure 1. Physiology of PCOS and the interactions of hyperinsulinemia, obesity, and hyperandrogenism. SHBG, sex hormone-binding globulin; LH, luteinising hormone; FSH, follicular stimulating hormone; T, testosterone; E, oestrogen. Reprinted from Dumesic DA et al. Scientific statement on the diagnostic criteria, epidemiology, pathophysiology, and molecular genetics of polycystic ovary syndrome. *Endocrine Reviews.* 2015; 36(5):487-525, with permission from the Oxford University Press.

Prenatal exposure

In addition to the genetic, ethnic, and environmental factors that predispose to PCOS, it is possible that various factors affecting the female foetus in the intrauterine life contribute to PCOS development later in life. About 30 years ago, Barker and colleges published studies implying that undernutrition during foetal life and poor infant growth were followed by a number of different metabolic abnormalities and coronary heart disease later in life.[29] This hypothesis has been developed further over the years, to the concept of 'Developmental Origins of Health and Disease'.[30] This concept is based on the theory that events early in life during the development of the organs result in epigenetic and gene expression changes that programme the individual for an increased risk of chronic diseases later in life.[31]

Some human studies have suggested that high and low birthweight are associated with increased risk of developing PCOS,[16, 32, 33] while other studies have not confirmed such association.[34-36] Maternal body mass index (BMI) is positively correlated with maternal testosterone levels during pregnancy, and also with higher levels of amniotic fluid testosterone in the female foetus.[37, 38] Maternal smoking during pregnancy has been shown to affect the female foetus' ovaries, expressed as higher densities of primordial follicles,[39] and lower proliferation of germ cells and somatic cells.[40, 41] Further, exposure to increased levels of PCOS-related hormones during pregnancy might affect foetal growth and be associated with adverse pregnancy outcomes.[42] To the author's knowledge, no past studies have looked at the possible association of maternal obesity or smoking and the risk of developing PCOS later in life for the female foetus.

Testosterone

One foetal exposure that may affect the risk of developing chronic disease later in life is testosterone. Testosterone is produced in the adrenal glands and ovaries, but also in the placenta during pregnancy.[14] Androgens such as testosterone and androstenedione are converted into oestradiol by aromatase in the liver, adipose tissue, and skin.[13] It has been demonstrated that pregnant women with PCOS have higher levels of testosterone during the second trimester of pregnancy than women without PCOS.[43] This can perhaps be explained by increased testosterone production in the adrenal glands, ovaries, and/or placenta during pregnancy in women with PCOS compared with in women without PCOS. Also, the placenta in women with PCOS expresses higher activity of 3β-hydroxysteroid dehydrogenase and lower activity of aromatase, plausibly contributing to higher levels of testosterone.[44]

Increased levels of testosterone during foetal life have been suggested to be involved in the PCOS pathogenesis. Use of animal models have demonstrated that sheep,[45] monkeys,[46] rats[47, 48], and mice[49, 50] treated with testosterone or dihydro-testosterone prenatally exhibit ovarian and endocrine traits similar to those in women with PCOS, such as luteinising hormone hypersecretion, enlarged polyfollicular ovaries, and functional hyperandrogenism. It remains a matter of debate if this is the case also in humans. Aromatase in the human placenta converts androgens into oestrogens, protecting both the mother and foetus from excessive androgen levels.[51] According to Caanen et al., testosterone levels are higher in pregnant women with PCOS than in pregnant controls, at both 20 gestational weeks and delivery. Still, the levels in the umbilical cord in pregnancies with female foetuses were not different between PCOS women and controls, further emphasising the function and high activity of the placental aromatase, also in PCOS women.[52] However, this was a small study

with only 20 PCOS women, and further studies in humans are required. Conversely, Kallak et al. demonstrated higher levels of amniotic fluid testosterone in pregnancies with female foetuses where the mother's weight gain during pregnancy was more than one standard deviation above the cohort mean. This finding suggests that factors affecting maternal hormone levels are also of relevance for foetal exposure.[38]

Anti-Müllerian hormone

Anti-Müllerian hormone (AMH) is a glycoprotein that is important for male sexual differentiation during foetal life by causing regression of the Müllerian ducts, which otherwise form the female reproductive tract.[53] In males, AMH is produced by Sertoli cells in the testes. It can be measured in prepubertal boys to explore its functional activity.[54] In females, AMH is produced by granulosa cells in the preantral and small antral follicles in the ovary and it can be considered an endocrine marker for ovarian reserve.[55] It is typically, but not exclusively, elevated in women with PCOS.[56-60] Previous longitudinal studies have demonstrated that AMH levels in healthy fertile women decrease by approximately 50% during pregnancy and normalise quickly after delivery.[61, 62] The same trend of AMH levels during and after pregnancy is seen in women with PCOS.[63]

Foetal exposure to excess AMH induces PCOS-like phenotypic traits in mouse offspring.[42] Moreover, prenatal AMH exposure leads to increased maternal and offspring testosterone levels and diminishes placental aromatase function, all in all opening up for a viable route by which maternal and placental testosterone can pass over to the foetus.[42] Recent studies have demonstrated that early pregnancy loss is associated with lower levels of pre-pregnancy AMH.[64, 65] Further, some studies have shown that AMH levels are lower in women with gestational hypertension and preeclampsia,[66, 67] while Birdir et al. did not confirm such association.[68] However, studies on the association of AMH levels and pregnancy complications are scarce and further studies on this topic are needed.

PCOS and short- and long-term health outcomes

Women with PCOS often have problems with oligo- and anovulation, and hence greater risk for infertility. Since their ovarian reserve is good, pregnancy can frequently be achieved by ovulation stimulation.[69] In addition to infertility, women with PCOS often have insulin resistance and hyperinsulinemia. Insulin abundance can further stimulate ovarian production of androgens[70] and suppress production of SHBG[71], altogether leading to further hyperandrogenism. Women with type 1 diabetes mellitus have increased risk of developing

PCOS, probably caused by iatrogenic hyperinsulinemia through medication.[72] Women with PCOS frequently have comorbid metabolic disturbances such as type 2 diabetes, hypertension, and dyslipidemia.[73] The accumulation of diverse risk factors in women with PCOS increases their risk of cardiovascular diseases later in life.[74] The association of PCOS with endometrial hyperplasia and endometrial cancer is uncertain, but PCOS is associated with several risk factors of endometrial cancer such as oligo- and amenorrhea, hyperinsulinemia, and obesity.[75]

PCOS and pregnancy complications

Infertility is more common among women with PCOS than women without PCOS, as is pregnancy achieved with the help of ovulation stimulation.[76] During pregnancy, women with PCOS also face a higher risk of diverse complications such as miscarriage, preterm birth, and the development of gestational hypertension, preeclampsia, or gestational diabetes.[77, 78] An increased risk of having a small for gestational age (SGA) or a large for gestational age (LGA) infant has been reported in women who in addition to having PCOS are also obese.[77] This association of PCOS and pregnancy complications can be influenced by obesity, pregnancy achieved with assisted reproductive technology (ART), as well as hyperinsulinemia and hyperandrogenism in the PCOS women.[79] In a study from 2010, Palomba et al. described an increased overall risk of pregnancy complications in hyperandrogenic women with PCOS compared with normoandrogenic PCOS women.[80] In a Danish study by Naver et al. from 2014 on a PCOS population seeking help for infertility, increased risk for preeclampsia and preterm birth (<37 gestational weeks) was seen for hyperandrogenic PCOS women compared with women without PCOS, but not for normoandrogenic PCOS women.[81] Another Danish study by Mumm et al. from the same year, in a more general population, did not confirm differences in obstetric outcome between different phenotypes of PCOS.[82] Kollman et al. showed no difference in pregnancy complications between PCOS women with different phenotypes.[83] De Wilde et al. found increased risk for gestational diabetes in women with PCOS compared with women without PCOS, and the association was stronger for hyperandrogenic than normoandrogenic PCOS women.[84] Notably, in that study, only 14% of the PCOS women conceived spontaneously compared with all of the women in the non-PCOS group, which might introduce bias. Studies that investigate risk for pregnancy complications between different phenotypes or androgenicity of PCOS are limited in size, with the largest study including merely 294 PCOS women (Naver et al.)[81] with information on phenotype or androgenicity. Large study populations are important to detect significant associations, especially when evaluating rare outcomes. One limiting factor in conducting large studies stratifying PCOS

women by phenotype or androgenicity is that it is hard to retrieve such information from nationwide patient registers. Overweight and obesity and increased gestational weight gain might also be contributing factors in the increased risk of pregnancy complications in women with PCOS. Overweight and obese women have increased risk of PCOS-related pregnancy complications such as gestational diabetes,[85] preeclampsia[86] and preterm birth.[87] Women who exceed the recommended gestational weight gain (GWG), according to the Institute of Medicine guidelines from 2009[88], are at increased risk of caesarean delivery and children born LGA or macrosomic.[89] Some evidence suggests that GWG is higher in women with than without POCS,[90] particularly in normal weight and overweight women,[91] although other studies cannot confirm this finding.[92] Zhang et al. demonstrated that normal-weight women with PCOS and with excessive GWG had increased risk of LGA children compared with non-PCOS women, and PCOS women with BMI $\geq$ 25 kg/m^2 and excessive GWG had increased risk of caesarean delivery compared with non-PCOS women.[93]

Preterm birth

Definition

Preterm birth is defined as a birth before 37 gestational weeks and occurs in approximately 5–15% of all infants. The World Health Organization (WHO) has divided preterm birth by severity into extremely (22–27 gestational weeks), very (28–31 gestational weeks) and moderately (32–36 gestational weeks) preterm birth.[94]

Aetiology

The aetiology of preterm birth is multifactorial. The onset of preterm birth can be considered as being either spontaneous or medically indicated, and the aetiology can be expected to be different for those two types of preterm births. Classification of birth onset is regardless of delivery (vaginal or caesarean section).

Spontaneous start refers to spontaneous cervix shortening and opening with or without labour, and preterm prelabour rupture of membranes (PPROM). Around two thirds of preterm births start spontaneously and can be triggered by diverse pathophysiological mechanisms.[95] Factors considered to be involved in this process include overstretching of the myometrium, impaired maternal-foetal immune tolerance, decidual bleeding or senescence, vascular disease of the placenta, bacterial infection, or inflammation.[87, 96-98] Spontaneous preterm birth has been associated with increased levels of pro-inflammatory proteins such as interleukins 1[99] and 6[100] and tumour necrosis factor.[101] These proteins are associated with cervical ripening and may also cause both

weakening of the membranes and preterm myometrial contractions,[102] resulting in preterm birth. In addition, androgens might also be an important factor in the pathogenesis leading to spontaneous preterm births. It has been hypothesised that androgens may influence remodelling and ripening of the cervix, at term, and possibly also in preterm deliveries.[103] Studies have shown that administration of dehydroepiandrosterone sulfate induces cervical ripening,[104, 105] possibly through upregulating interleukin-8 and interleukin-8 receptor in cervical fibroblasts,[106] increasing maternal serum concentration of oestradiol 17-β,[107] and altering proteoglycan content in the extracellular matrix in the cervical tissue.[108] Further, treating hyperandrogenic women with the antiandrogen ethinyl oestradiol/cyproterone acetate prior to conception has been shown to decrease the risk for preterm delivery.[109]

Medically indicated preterm births are due to maternal or foetal conditions, such as preeclampsia, gestational hypertension, gestational diabetes, or foetal growth restriction.[77, 78]

Consequences

Preterm birth is associated with both infant morbidity and mortality.[110] With increasing severity of prematurity, the risk of adverse health outcome and long-term consequences for the infant increases.[111] Respiratory disorders, necrotising enterocolitis, retinopathy of prematurity, hearing impairment, and neuro-psychologic disturbances are among the morbidities that have been associated with preterm birth.[110]

Preterm births are also a cost burden for society. Preterm-born children often need medical care for weeks or months at a neonatal unit and in some cases continued medical care in the long term for diverse problems such as cerebral palsy, impaired hearing or vision, and possibly mental retardation.[112, 113] The family of the preterm child can also suffer high costs, both medical costs and indirect costs such as the mother staying away from work for a longer period after the birth or working part-time longer than originally planned.[114]

PCOS and preterm birth

In women with PCOS, the adipose tissue-related chronic systemic low-grade inflammation[115, 116] might increase the risk of spontaneous preterm birth.[96, 117] This applies also for lean women with PCOS; the increased inflammation is found in women with normal BMI or after adjustment for BMI.[118-120] This might be explained by different properties of the adipose tissue in women with PCOS compared with in women without PCOS. A study on mice shows increased adipocyte hypertrophy with increased pro-inflammatory macrophage accumulation in PCOS phenotype dams compared with controls.[121] A pro-inflammatory state in the liver with increased expression of the cytokine interleukin 1β in the PCOS dams[121] would suggest that the source of increased

inflammation may also be related to tissues other than adipocytes. Further, women with PCOS also have increased risks of pregnancy complications that might lead to medically indicated preterm birth, such as pre-eclampsia, gestational hypertension, and gestational diabetes.[122-125]

Previous studies have already shown that women with PCOS are at increased risk of delivering preterm.[125, 126] Study size has been a limitation when investigating the association between PCOS and preterm delivery, leaving a number of pertinent questions unanswered. For this reason, it is presently unknown if PCOS is associated with both spontaneous and medically indicated onset of preterm birth, and if this differs between severities of prematurity. Previous studies have indicated that the risk of very preterm birth (<32 weeks) might be higher than the risk of moderately preterm birth (32–36 weeks).[126, 127] To the author's knowledge, no study has examined the risk of extremely preterm birth in women with PCOS, but this is an important topic, since infant morbidity and mortality increase with decreasing gestational age at birth. As for onset of preterm births in women with PCOS, to the author's knowledge, only two previous studies have reported on spontaneous preterm births and medically indicated cases separately. Yamamoto et al. found similar rates of spontaneous preterm births in women with and without PCOS.[127] De Wilde et al. did not confirm increased risk for spontaneous preterm birth in women with PCOS in an adjusted model of odds ratios (ORs), but found an increased risk of medically indicated preterm birth in the PCOS group.[84] Since spontaneous and medically indicated preterm births supposedly have differing pathophysiologies, it is important to separate these entities when studying association with PCOS.

Stillbirth

Definition

Stillbirth is defined by WHO as the death of a foetus at or after 22 gestational weeks or of a foetus that has reached a birthweight of 500 g. Stillbirth can further be categorised into late foetal death (foetal weight greater than 1000 g or gestational age after 28 weeks) and early foetal death (500–1000 g or gestational age 22–28 weeks).[128]

Aetiology

Several risk factors for stillbirth have been identified. In high-income countries, the most common possibly modifiable risk factors are maternal overweight and obesity, high maternal age, and maternal smoking. Pre-existing maternal diseases, such as diabetes and hypertension, are associated with stillbirth and even emerging complications during the pregnancy such as preeclampsia and foetal growth restriction.[129] Both foetal growth restriction

and placental abruption are risk factors for stillbirth, indicating that placental pathology or placental dysfunction contribute to stillbirth.[129] Routine checks in maternity care, such as symphysis–fundus measurements, aim to identify presumably growth-restricted foetuses. Growth-restricted foetuses are at increased risk of prenatal and intrapartum hypoxia and, in the worst case, stillbirth.[130] Close monitoring of the foetus is crucial in maternal care of women with growth-restricted foetuses. The aim is to prevent prematurity, but still induce birth when there is a high risk for stillbirth. Other important known causes of stillbirth are infections and foetal anomalies.[131] Stillbirth remains unexplained in 25–60% of cases occurring after 28 weeks of gestation; the frequency varies greatly because of different classification systems between studies.[131]

Consequences

Though stillbirths are rare, they are a devastating occurrence for the family. Long-term consequences, such as depression, anxiety, or post-traumatic stress disorder, are known among mothers, and fathers can also experience negative psychosocial effects.[132] Stillbirths can affect subsequent parenting[133] and result in termination of marriage.[134]

PCOS and stillbirth

There is a limited number of studies on the association of PCOS and stillbirth. In a retrospective case-control study in Australia, PCOS has been mentioned as a risk factor for stillbirth, defined as birth of a stillborn child at 20 weeks of pregnancy or later, or $\geq$ 400 g if gestational age was unknown. However, the number of women with PCOS in the study was only 16. PCOS was more common in the stillbirth group than in the control group (8.5% and 1.9%, respectively, $p<0.05$). The OR for stillbirth in women with PCOS compared with controls was 5.36, but the confidence interval (CI) was very wide (0.80–35.92), showing the problem of power in studies on this topic.[135] Another Australian study, a matched cohort study, demonstrated higher incidence of stillbirth in women with PCOS than in controls (1.8% vs 0.6%, $p<0.001$), but again the risk was not statistically increased (OR 1.23, 95% CI 0.80–1.89).[136] A Swedish register-based study including births between 1995 and 2007, with over 3,700 births in PCOS women, showed no association of PCOS with stillbirth (adjusted OR 0.90, 95% CI 0.50–1.63).[126] In that study, the definition of stillbirth was a death of a foetus at or after 28 gestational weeks. From July 2008, stillbirth has been defined as death of a foetus at or after 22 gestational weeks in Sweden. This enables a register-based study from 2008 and onwards in Sweden to include a larger proportion of all stillbirths. Further, since stillbirth is a very rare occurrence in pregnancy, a large study population is needed to examine this outcome, and a longer time for follow-up would include more women with PCOS.

Intergenerational studies and birth cohorts

Evidence suggests that genetic variants are transferred across generations, as are behavioural, environmental, and socioeconomic characteristics. Over the last two decades, a rising number of studies has focused on the hypothesis that exposure to risk factors in early life or the foetal period have consequences for adult health and diseases. Still, while interpreting such studies, it is important to separate causal and non-causal associations. Intergenerational studies investigate the same characteristic through two or more generations. They can include studies that investigate if characteristics in one generation are associated with other characteristics in another generation. Many intergenerational studies focus on establishing associations, while others elucidate the mechanisms underlying such associations.[137]

Birth cohort studies enable systematic prospective collection of data and mothers are easily approached during antenatal care and delivery ward. These studies can have large cohort sizes and enough power to examine rare outcomes, like extremely preterm birth and stillbirth. Prerequisites for good birth cohort studies are well-functioning, population-wide health registers and unique personal identification numbers which enable tracing of individuals in several different registers.[137, 138]

Aims

The overall aim of this thesis was to gain further knowledge of the association between the intrauterine environment and the development of PCOS in offspring, as well as of the association between maternal PCOS and adverse pregnancy outcomes.

The specific aims of the studies were:

I To explore if prenatal exposures and infant size at birth were associated with PCOS diagnosis in female offspring.

II To study the associations of AMH levels in women with PCOS during pregnancy with testosterone levels and adverse pregnancy outcomes.

III To examine the association of maternal PCOS and risk of preterm birth by severity and type of onset of preterm birth, with a specific aim to explore extremely preterm birth.

IV To investigate the association of maternal PCOS and risk of stillbirth. Further, to study if PCOS-women with more severe hyperandrogenism had stronger association.

Material and methods

Overview of the studies

This thesis consists of four studies with differing study designs, described in Table 1.

Table 1. *Overview of the studies.*

Paper	Study population	Study design	Exposure	Outcome
I	681,123 females born in Sweden 1982–1995	Observational register-based cohort study	Prenatal exposure and size at birth	PCOS diagnosis in offspring
II	479 pregnant women in Uppsala county 2007–2015	Matched cohort study	AMH, PCOS	Levels of testosterone and SHBG, pregnancy complications, size at birth
III	1,046,448 pregnancies in Sweden 2005–2014	Observational register-based cohort study	PCOS diagnosis	Preterm birth, by severity and type of onset
IV	283,433 pregnancies in Sweden 1997–2015	Observational register-based cohort study	PCOS diagnosis, PCOS by androgenicity	Stillbirth

Data sources

National registers

The Swedish National Board of Health and Welfare gave access to information from the Swedish Medical Birth Register (MBR), the National Patient Register, the Cause of Death Register and the Swedish Prescribed Drug Register. Statistics Sweden provided data from the Swedish Register on Participation in Education and the Total Population Register. Individual record linkage between the registers was possible through each individual's unique personal registration number, which is assigned to Swedish residents at birth or immigration.[138]

Swedish Medical Birth Register

The MBR contains data on over 98% of all births in Sweden since 1973 and includes prospectively collected demographic data, information on reproductive history, and complications that occur during pregnancy, delivery, and the neonatal period.[139] In Sweden, antenatal care is standardised and free of charge. During the first antenatal visit, usually taking place at the end of the first trimester, the mother is interviewed about her medical and obstetric history, including her height (self-reported) and current smoking habits. Weight is instructed to be measured in the first trimester with the woman wearing light indoor clothes. Data on some chronic diseases (such as pre-gestational diabetes) and smoking habits are recorded in checkboxes. Complications during pregnancy and delivery are classified in accordance with the International Classification of Diseases (ICD) and noted by the responsible obstetrician at discharge from the delivery hospital. Information on each pregnancy and delivery is forwarded to the MBR through copies of standardised antenatal, obstetric, and paediatric records.

Mothers' height and weight were registered in the MBR 1982–1989 and from 1992 and onwards, enabling the calculation of early pregnancy BMI. Data on smoking habits in early pregnancy and years of involuntary childlessness before the index pregnancy are included in the MBR since 1982.[140] Gestational diabetes was first identified with an ICD code in the ICD-9 version, available from 1987.[141]

Some of the variables in the studies, such as diseases and complications during pregnancy, were defined using ICD codes. ICD version 7 was applicable in Sweden between 1964 and 1968, version 8 between 1969 and 1986, version 9 between 1987 and 1996, and version 10 from 1997.[141]

Swedish National Patient Register

The National Patient Register includes information on dates of hospital admissions and diagnoses since the 1960s, with full national coverage since 1987.[142] Since 2001, the register also covers specialised outpatient visits. Diagnoses are classified based on ICD codes.

Swedish Cause of Death Register

The Swedish Cause of Death Register comprises data on all deaths of people registered in Sweden since 1952.[143]

Swedish Register on Participation in Education

This is a register with information on the education of the Swedish population. The first version described the conditions on 31st December 1985 and annual

updates have been carried out since. It contains information on educational level in years and educational orientation.

Swedish Total Population Register

This register was created in 1968 and contains statistical information about the population composition, for example country of birth.[144]

Swedish Prescribed Drug Register

This register includes information on all dispensed prescribed drugs in Sweden from July 2005 and onwards. It contains information on the date of prescriptions of medicines and dispensation of them, linked to the patients' personal registration numbers. It also includes information on the kind of clinic where the medicine was prescribed and the education code for the prescriber.[145]

Uppsala Biobank of Pregnant Women

The Uppsala Biobank of Pregnant Women is a collection of venous blood samples from pregnant women in Uppsala county, Sweden. Since 31 May 2007, all Swedish-speaking pregnant women, aged 18 years and older, without blood-borne diseases, attending the second-trimester routine ultrasound scan (gestational weeks 16–19) in Uppsala county have been invited to participate in the biobank, if a research nurse was available at the time of the scan. The biobank is considered population-based as 97% of the pregnant population participates in the routine ultrasound examination, and as all routine ultrasound examinations in Uppsala county are performed at Uppsala University Hospital. Further, Uppsala University Hospital is the only available delivery ward within the county, leading to excellent follow-up of the participants.[146] It is estimated that up to 50% of the pregnant population in Uppsala county participate in the biobank.[147] Upon inclusion, a blood sample and brief demographic data are collected, including data on previous and ongoing chronic disorders, ongoing medication, smoking in early pregnancy, height, and pre-pregnancy weight. The blood samples are collected in EDTA-containing tubes and centrifuged at 1,500 *g* for 10 min. Plasma and buffy coat are separated within two hours and stored at -70 °C.

Study populations and study designs

Paper I

Paper I was a national register-based cohort study of the associations between prenatal exposures or size at birth and the risk of developing PCOS later in

life. The study population was all singleton, live-born females between 1 January 1982 and 31 December 1995 who reached at least 15 years of age. Births included in the MBR during this period totalled 1,505,061. A total of 823,938 births were excluded because of multiple pregnancies (n=34,821), male gender or unknown sex of the child (n=755,838), stillborn (n=2,428), incorrect or missing personal identification number (n=2,569), death before 15 years of age (n=4,547), emigration before 15 years of age (n=23,643), and PCOS diagnosis before 15 years of age (n=92). A total of 681,123 births were eligible for analysis.

The research group collected data on maternal height, weight, smoking habits, age at delivery, parity and involuntary childlessness before the index pregnancy, presence of preeclampsia or diabetes during the index pregnancy, and the offspring's size and gestational age at birth from the MBR. Early pregnancy BMI was calculated, using the equation weight (kg)/height $(m)^2$; the same formula was used to calculate BMI in Papers II–IV. Pregnancies affected by preeclampsia and pre-gestational diabetes were identified using ICD codes and pre-gestational diabetes was further identified using the checkbox from the first antenatal visit. Data on maternal education in 2010 and maternal country of birth were collected from the Education Register and the Total Population Register, respectively. Maternal PCOS diagnosis was retrieved from the National Patient Register and defined as ICD codes: ICD-7, 275.20, ICD-8, 256.90, and ICD-9, 256E. In the Swedish versions of ICD-7 and ICD-8, the codes corresponded to what was formerly called Stein-Leventhal syndrome. For versions ICD-9 and ICD-10, the codes are specified as PCOS and Stein-Leventhal syndrome.

Several definitions were used to estimate offspring size at birth. First, offspring were categorised by birthweight (<2,500, 2,500–4,499, >4,500 g). Second, they were categorised by standardised birthweight based on gestational age and sex-specific Swedish birthweight curves.[148] Appropriate for gestational age (AGA) described infants whose weight was from the 10^{th} to the 90^{th} percentile for the gestational age. SGA were infants with birthweight below the 10^{th} percentile for the gestational age and LGA were infants with birthweight above the 90^{th} percentile. Third, the ponderal index, defined as birthweight/$height^3 \times 100$, was calculated. Low and high ponderal index were defined as a ponderal index score among the 10% lowest or highest in the study population, respectively. Lastly, the head circumference was calculated for gestational age. Small and large head circumference for gestational age were defined as more than 10% below or 10% above the mean head circumference for gestational age, respectively, based on the Swedish reference curve for new-born infants.[149]

Outcome

In this study, outcome was PCOS diagnosis at 15 years or later, obtained from the National Patient Register and identified using ICD-10 code E28.2. The population could be followed to a maximum of 28 years of age at the end of 2010.

Paper II

Paper II was a matched cohort study. The main aim was to evaluate the associations of AMH levels with testosterone levels and with pregnancy complications in women with PCOS. This cohort study included women from the Uppsala Biobank of Pregnant Women. Blood samples were collected from women that accepted to participate in the biobank, when they attended to the second-trimester routine ultrasound at Akademiska University Hospital, from 31 May 2007 and onwards. The research group identified all women diagnosed with an ICD-10 diagnosis of PCOS (E28.2) between 2003 and 2015 in electronic medical charts at the Uppsala University Hospital. The PCOS diagnosis was made based on Rotterdam criteria. The medical records of all women with PCOS were scrutinised to verify the PCOS diagnosis and to obtain information on obstetric and perinatal outcomes. By September 2015, 174 pregnant women with PCOS had been included in the biobank. Fifteen women with PCOS were excluded; four women due to twin pregnancies, two due to late miscarriage or stillbirth, seven due to deliveries outside Uppsala county, and two as they were misdiagnosed with PCOS, leaving 159 women with PCOS available for hormonal analyses. The majority of women (n=99) had received the PCOS diagnosis as part of an infertility work-up, whereas the remaining women had consulted a physician for menstrual disturbances or hirsutism.

For each pregnant woman with PCOS, two BMI-matched controls with singleton pregnancies were chosen (n=320). Each control had donated a blood sample to the biobank during the same week as the respective PCOS woman and was otherwise healthy according to a self-report collected upon inclusion.

AMH results were available in all but three women (due to insufficient plasma samples) in the study population. Results were expressed in pmol/L. Plasma AMH levels were measured using the fully automated Acces Dxi sandwich immunoassay (B13127, Beckman Coulter). This assay measures the proAMH and the cleaved $AMH_{N,C}$ complex and uses recombinant human AMH as a calibrator. The limit of quantification of the assay is 0.57 pmol/L, with intra- and inter-assay imprecision less than 5%.

Outcome

The primary outcomes in this study were testosterone and SHBG levels, FAI, and PCOS-related pregnancy complications. Testosterone levels were missing in seven women due to insufficient amounts of sample for analysis. Testosterone was analysed using supercritical fluid chromatography (Waters ACQUITY® UPC²™, Milford, MA, USA) coupled with tandem mass spectrometry (XEVO® TQ-S, Milford, MA, USA), as previously described by Kallak et al.[38] The analysis was performed with an Acquity UPC² BEH column (150 mm, 3.0 mm, 1.7 µm at 40 °C [Waters, Milford, MA, USA]). Waters MassLynx version 4.1 software was used to acquire and analyse data. Masslynx NT4.1 software (Waters Corp., Milford, MA, USA) was used to obtain all data collected in centroid mode. Each sample was analysed twice and the average values were reported (CV<3%). The linearity of the method was evaluated over a range of concentrations (0.1–50 nmol/L) and the correlation coefficient (R^2) was 0.998.

SHBG was also analysed in the plasma samples from the biobank. Analysis was not possible in one of the samples for technical reasons. Immuno-kits for SHBG were obtained from R&D Systems (Minneapolis, USA) with the catalogue number DSHBG0B. The assay was of the sandwich type, using a precoated 96-well plate and a supply of enzyme-labelled secondary antibody as well as washing buffer, assay buffer, substrates, and standard. The resulting absorbance was read in a BioRad Model 680 Microplate Reader at 450 nm with 595 nm as background. Repeatability of the assay was checked and the median repeatability standard deviation RSD for SHBG was 13.5%. The accuracy is presented by the manufacturer using an in-house preparation calibrated against NIBSC/WHO International Standard 08/266. FAI was calculated using the formula: testosterone (nmol/l)/SHBG (nmol/l).

Information on maternal age, parity, height, weight in early pregnancy, country of birth, smoking during early pregnancy and reproductive history was derived from the standardised antenatal records. Information on pregnancy complications and perinatal outcomes was derived from the standardised obstetric and paediatric medical records. Obstetric diagnoses according to ICD-10, recorded in the obstetric medical records, were noted. Obstetric and neonatal outcomes of interest were preeclampsia, gestational hypertension, gestational diabetes, preterm birth, and infant size at birth. Preterm birth was defined as delivery prior to 37+0 gestational weeks. SGA and LGA were defined as having a birthweight of more than two standard deviations below or above, respectively, the mean birthweight for gestational age and sex according to the reference curve.[148] The same definitions of SGA and LGA were used in Papers II and III.

Paper III

Paper III was a national register-based cohort study on associations between PCOS and preterm birth. The study population was women with singleton pregnancies giving birth to a live infant in Sweden in the years 2005–2014. The cohort was established from the MBR. Pregnancies with unknown gestational age at birth (n=484) or unknown personal identification number (n=14,115) were excluded. Women with gestational age more than 43 weeks and 6 days were also excluded, as it was presumed that the gestational age was incorrectly registered (n=692). A total of 1,046,448 women were eligible for analysis. Information on maternal age, parity, height, weight in early pregnancy, daily smoking in early pregnancy, cohabitation, reproductive history, presence of hypertensive or diabetic diseases, country of birth, years of education, and year of delivery was gathered from appropriate registers. Hypertensive disease was classified into 1) no hypertensive disease, 2) chronic hypertension, and 3) pregnancy-induced hypertension (including gestational hypertension, preeclampsia, and eclampsia). Diabetic diseases were classified into no diabetic disease, pre-gestational diabetes (diabetes type 1 and 2), and gestational diabetes.

The exposure in the study was PCOS diagnosis before or during the index pregnancy and was identified in the National Patient Register. PCOS was defined using ICD codes: ICD-8, 256.90, ICD-9, 256E and ICD-10, E28.2. The Rotterdam criteria for PCOS were swiftly introduced into clinical practice in Sweden from 2003 and it has been assumed that they were generally established from 2005, the year initiating the study period.

Outcome

The primary outcome was preterm birth by degree of severity, with special interest in extremely preterm birth. Preterm birth was defined as gestational length <37 weeks and was categorised into extremely preterm birth (22w0d–27w6d), very preterm birth (28w0d–31w6d), or moderately preterm birth (32w0d–36w6d). The secondary outcome was preterm birth by onset of delivery, categorised into spontaneous (spontaneous onset of labour or PPROM [ICD-10, O42]) or medically indicated preterm birth (vaginally induced onset or caesarean delivery before onset of labour). Information on mode of onset of delivery was missing in 4,409 women, 67 with PCOS and 4,342 without PCOS.

In Sweden, all pregnant women are offered a first or early second-trimester ultrasound free of charge, at which dating of the pregnancy is performed. In this dataset, gestational age was determined using the following hierarchy:

first-trimester or early second-trimester ultrasound (90.8%), date of last menstrual period reported at the first prenatal visit (4.4%), and postnatal assessment (4.7%).

Paper IV

Paper IV was a national register-based cohort study on the association between PCOS and stillbirth. All women born 1950–1999, with PCOS diagnosis in the National Patient Register 1997–2017, were identified. PCOS was defined using the following ICD-10 codes: E28.2 (PCOS), N97.0 (anovulatory infertility), and E28.1 (hypersecretion of ovarian androgens), n=53,842 in the National Patient Register. A reference group was identified among women without a registered PCOS diagnosis, including five women for each PCOS woman, n=269,210. For both groups, pregnancies during the years 1997–2015 were identified in the MBR. Women without pregnancies during this period were excluded (n=27,633 in the PCOS group, n=133,008 in the non-PCOS group). Women with the diagnosis of congenital adrenal hyperplasia (n=30) were excluded from the PCOS group, as were women with diagnoses of both anovulatory infertility and primary ovarian insufficiency (n=166) or hyperprolactinemia (n=7), since their anovulatory infertility was unlikely caused by PCOS. Multiple pregnancies were excluded from both groups (n=2,814 in the PCOS group, n=6,477 in the non-PCOS group). Further, pregnancies with missing information on gestational length were excluded (n=16 in the PCOS group, n=115 in the non-PCOS group), as were pregnancies with gestational length over 43 weeks + 6 days (n=8 in the PCOS group, n=31 in the non-PCOS group). Lastly, pregnancies after ovulation stimulation were excluded from the non-PCOS group (n=1,626), as they might represent undiagnosed PCOS cases. The final cohorts consisted of 41,827 births in women with PCOS and 241,606 births in non-PCOS women.

A wider definition was used for PCOS in this study than in the earlier studies. The rationale for including women with anovulatory infertility in the PCOS group was that 80–90% of these women have PCOS, according to the Rotterdam criteria.[150, 151] Further, it is possible that during infertility assessment at a fertility clinic, women with PCOS only get the diagnosis of anovulatory infertility registered, not the PCOS diagnosis.

In this study, the research group wanted to further identify PCOS women with hyperandrogenic symptoms requiring medical treatment, expected to have more severe form of hyperandrogenism. The aim was to investigate if they had stronger association with stillbirth than PCOS women without anti-androgen treatment, compared with non-PCOS women. By linkage to the Prescribed Drug Register, women with at least two dispensations of a prescription for an

anti-androgenic drug during the years 2005–2017, either before, during or after the index pregnancy, were identified. The anti-androgenic drugs included in this study were: spironolactone (ACT code C03DA01), finasteride (D11AX10), eflornithine (D11AX16), finasteride and dutasteride (G04CB), flutamide (L02BB01), bicalutamide (L02BB03), and the combined oral contraceptive containing ethinyl oestradiol (EE) and cyproterone acetate (G03HB01). In addition, the following anti-androgenic combined oral contraceptives were included: pills with EE and desogestrel (G03AA09), EE and drosperinone (G03AA12), and EE and dienogest (G03AA16). A total of 4,223 PCOS women with anti-androgen medical treatment (10.1%) were identified and the remaining PCOS women were without anti-androgen treatment.

Information on covariates was gathered in the same way as described for Study III, except for diabetic diseases. In this study, diabetic diseases were categorised as type I diabetes, type II diabetes, unspecified pregestational diabetes, or gestational diabetes.

Outcome

The outcome was stillbirth, identified in the MBR. In the MBR, stillbirths were registered from gestational week 28+0 until July 2008. From July 2008 and onwards, stillbirths were registered from gestational week 22+0. Stillbirths can have occurred either prior to or during labour.

Statistical methods

Paper I

To explore the association between prenatal exposures and size at birth and risk of a PCOS diagnosis in the offspring, Cox regression analysis was used to estimate hazard ratios (HRs) and adjusted hazard ratios (aHRs) with 95% CI. A test of Schoenfeld residuals was performed and showed fitness for the variables in the model. Adjustments were made for all maternal characteristics, both exposures (BMI, smoking habits, diabetes, and preeclampsia) and covariates (maternal age, parity, years of involuntary childlessness, educational level, country of birth, and PCOS diagnosis), and for size at birth, when testing each exposure in turn.

The statistical software package SAS 9.4 (Statistical Analysis Software version 9.4; SAS Institute Inc., Cary, NC, USA) was used for analysis.

Paper II

Clinical characteristics and obstetric and neonatal outcomes were compared between women with PCOS and healthy controls using independent t-tests or chi-squared tests.

Comparisons of second-trimester hormone levels between controls and normo- and hyperandrogenic PCOS women were performed using one-way analysis of variance with post-hoc Tukey HSD tests or Kruskal-Wallis test followed by Mann-Whitney U-tests, depending on whether the distribution was normal or not.

Correlations between AMH and testosterone, FAI, age, and BMI were analysed using Spearman's rank correlations, since AMH and testosterone were not normally distributed. To determine independent factors influencing AMH levels, multiple linear regression models were used.

A multivariable logistic regression model was used to analyse the association of AMH levels and obstetric outcomes and size at birth. Adjustments were made for age, parity, BMI, maternal height, smoking, country of birth, gestational length at blood sampling, and year of delivery.

All statistical analyses were performed using SPSS software (version 24.0, IBM Corp., Armonk, NY).

Paper III

Associations between maternal PCOS diagnosis and risk of preterm birth were estimated using univariate and multivariate logistic regression. Crude ORs and adjusted odds ratios (aORs) with 95% CIs were calculated. As observations were not independent in women who delivered more than once during the study period, the generalised estimation equation method was applied in the analyses. To obtain a systematic representation of any causal relationship between PCOS diagnosis and preterm birth, a directed acyclic graph (DAG) was constructed,[152] utilising the DAGitty web application from *www.dagitty.net*. The DAG was then used to determine which covariates should be considered to be confounders. Adjustments were made in two steps. First, adjustments were made for maternal age, parity, smoking, cohabitation, country of birth, and year of delivery. In a secondary step, adjustments were made for the same variables and BMI, since it is debatable whether or not adjustment should be made for BMI. High BMI may be part of the PCOS condition and can be seen as a mediator in the association between PCOS and preterm birth. Increasing BMI is also associated with a higher risk of present-

ing with PCOS symptoms[26] and can thereby be considered a confounder. Covariates were included as categorical in the statistical models, and cases with missing data were excluded.

When risk for moderately preterm birth was calculated, all women with extremely preterm birth were excluded, since they could not be considered still at risk. Similarly, when calculating the risk for moderately preterm birth, women with extremely or very preterm birth were excluded from the analyses. The research group also wanted to investigate whether the preterm risk in PCOS women was mediated by PCOS-related disorders, like hypertension and diabetes. Therefore, the risk of preterm birth in PCOS women was calculated after exclusion of women with hypertensive or diabetic diseases (both pre-gestational and gestational disorders). As a sensitivity analysis, the risk of preterm birth by severity was calculated in a restricted population with only primiparous women.

Statistical analyses were performed using SAS version 9.4 (Statistical Analysis Software version 9.4, SAS Institute Inc., Cary, NC, USA) and SPSS software (version 25.0, IBM Corp., Armonk, NY).

Paper IV

Associations between maternal PCOS diagnosis and risk of stillbirth were estimated using univariate and multivariate logistic regression. Crude ORs and aORs were calculated. The generalised estimation equation was applied in the analyses. A DAG was built to get a systematic representation of causal relationship of PCOS diagnosis and stillbirth and to decide which covariates should be considered to be confounders. Adjustments were made in two steps in the multivariate logistic regression model. First, the OR was adjusted for maternal age, parity, type 1 diabetes, educational level, and country of birth, and in a second adjusted analysis, also for BMI. Covariates were included as categorical or continuous in the statistical models, and cases with missing data were excluded. Thereafter, women with PCOS were stratified into those with and those without anti-androgen medical treatment, and ORs and aORs for stillbirth were estimated, using non-PCOS women as the reference group.

Stillbirth can occur at various times during pregnancy. To be able to also take into account the time at risk until stillbirth occurs, when estimating the association between PCOS and stillbirth, analyses with a Cox regression model were performed. In the Cox regression model, observations must be independent of each other, meaning that only one pregnancy for each woman may be included. The analysis was therefore conducted on primiparous women only.

The research group also constructed a plot of stillbirth risk by gestational age, stratified by PCOS. The risk for specific gestational age was defined as the number of stillbirths during a time interval (from gestational week 22 and then in 2-week intervals), divided by the total number of foetuses undelivered at the beginning of the interval. The proportion was multiplied by 10,000 to provide a gestational age-specific stillbirth rate per 10,000 undelivered foetuses.

To quantify the proportion of stillbirths where PCOS might be a contributing factor, the group calculated the population attributable fraction (PAF) for PCOS. Multivariate logistic regression was used to estimate aORs with 95% CIs. To calculate PAF, the following formula was used: $PAF = pd_i\ [(Or_i - 1)/OR_i]$.[153] The pd_i value represents the proportion of total cases in the population arising from the $_i$th category – in this case, women with PCOS.

All statistical analyses were performed using SPSS software (version 27.0, IBM Corp., Armonk, NY).

Ethical statement

Ethical reviews were conducted for all four studies and all data were stored, coded or pseudonymised, on secure servers. Study I was approved by the regional ethical committee board in Stockholm, Sweden (2008/1182-31/4 and 2011/1856-32, date of approval November 21 2011). Study II was approved by the regional ethical review board in Uppsala, Sweden (diary number 2007/181, date of approval August 07 2007, and 2017/029, date of approval March 22 2017) and written informed consent was obtained from all women upon acceptance to participate in the biobank. Study III was approved by the regional ethical committee board in Stockholm, Sweden (2008/1182-31/4 and 2011/1856-32, date of approval November 21 2011). Study IV was approved by the regional ethical review authority in Uppsala, Sweden (diary number 2017/309, date of approval August 09 2017).

Results

Paper I

Of 681,123 females eligible for analysis, 3,738 (0.54%) were diagnosed with PCOS during the follow-up period. Females who developed PCOS more often had mothers with BMI in the higher categories, who smoked during pregnancy, were younger, primiparous, and had more years of involuntary childlessness before the index pregnancy than females without PCOS. Mothers of the PCOS females also more often had a lower educational level, were more often born in non-Nordic countries, and had more often been diagnosed with PCOS themselves (1.2% compared with 0.2% among the mothers of females without PCOS diagnosis). Pre-gestational diabetes, gestational diabetes, or preeclampsia were not more common in the mothers of offspring with PCOS.

Females with mothers who were overweight or obese had higher risk of PCOS diagnosis than females with mothers of normal weight (aHR 1.52, 95% CI 1.36–1.70 and aHR 1.97, 95% CI 1.61–2.41, respectively). If the mother smoked during pregnancy, the female offspring had an increased risk of PCOS compared with if the mother did not smoke; the risk increase was dose-dependent (1–9 cigarettes/day, aHR 1.31, 95% CI 1.18–1.47; ≥ 10 cigarettes/day, aHR 1.44, 95% CI 1.27–1.64). There were no associations between maternal age, parity, involuntary childlessness, pregestational diabetes, gestational diabetes, or preeclampsia in the index pregnancy and development of PCOS in the offspring (Table 2).

Table 2. *Risks of PCOS diagnosis by maternal characteristics, among singleton females born between 1982 and 1995 in Sweden.*

	PCOS diagnosis in female offspring		
		Hazard Ratio (95% CI)	
	Rate (%)	Crude	Adjusted[a]
Maternal body mass index in early pregnancy (kg/m²)[b]			
≤18	0.6	0.93 (0.78–1.10)	0.89 (0.74–1.06)
18.5–24.9	0.5		Reference
25.0–29.9	0.7	1.60 (1.44–1.78)	1.52 (1.36–1.70)
≥30.0	0.8	2.31 (1.93–2.76)	1.97 (1.61–2.41)
Maternal daily smoking in pregnancy			
No	0.5		Reference
1–9 cigarettes/day	0.7	1.27 (1.16–1.38)	1.31 (1.18–1.47)
≥10 cigarettes/day	0.8	1.48 (1.34–1.63)	1.44 (1.27–1.64)
Maternal age at birth (years)			
13–19	0.8	1.33 (1.12–1.57)	1.01 (0.79–1.29)
20–24	0.6	1.17 (1.08–1.27)	1.04 (0.93–1.16)
25–29	0.5		Reference
30–34	0.5	0.95 (0.87–1.04)	0.90 (0.80–1.01)
≥35	0.5	1.05 (0.94–1.17)	0.99 (0.85–1.16)
Parity at index pregnancy			
0	0.6		Reference
≥1	0.5	1.07 (1.00–1.14)	1.03 (0.94–1.14)
Involuntary childlessness before index pregnancy (years)			
<1	0.5		Reference
1–2	0.7	1.12 (0.95–1.31)	1.05 (0.86–1.29)
<2	0.7	1.23 (1.02–1.48)	1.14 (0.90–1.45)
Maternal education (years)			
≤12	0.4	1.31 (1.22–1.40)	1.10 (1.00–1.22)
>12	0.6		Reference
Maternal country of birth			
Nordic	0.5		Reference
Non-Nordic	0.8	2.07 (1.87–2.29)	2.00 (1.73–2.32)
Maternal diabetes during index pregnancy			
No	0.6		Reference
Pre-gestational	0.6	0.99 (0.64–1.47)	0.61 (0.29–1.29)
Gestational[c]	0.5	1.64 (0.97–2.77)	0.87 (0.32–2.33)
Maternal preeclampsia during index pregnancy			
No	0.6		Reference
Yes	0.6	1.17 (0.96–1.43)	1.05 (0.80–1.38)
Maternal PCOS diagnosis[d]			
No	0.2		Reference
Yes	1.2	8.02 (5.97–10.76)	6.59 (4.40–9.89)

[a] Adjusted for all the other variables in the table (maternal age, parity, years of involuntary childlessness before index pregnancy, presence of diabetes or preeclampsia at index pregnancy, as well as for maternal educational level, country of birth, and PCOS diagnosis) and for infant birthweight for gestational age.
[b] Information on maternal BMI was available between 1982–1989 and 1992–1995.
[c] Information on gestational diabetes was available for 1987 and later.
[d] During the years 1941–2010.

Being born SGA and having a small head circumference for gestational age were both associated with a later diagnosis of PCOS in crude estimates (HR 1.23, 95% CI 1.11–1.37 and HR 1.16, 95% CI 1.05–1.28, respectively), but not after adjustments (aHR 1.09, 95% CI 0.94–1.26 and aHR 1.04, 95% CI 0.90–1.19, respectively) (Table 3).

Table 3. *Risks of PCOS diagnosis by size at birth among singleton females born between 1982 and 1995 in Sweden.*

	PCOS diagnosis in female offspring		
		Hazard ratio (95% CI)	
	Rate (%)	Crude	Adjusted[a]
Birthweight for gestational age[b]			
Small (SGA)	0.7	1.23 (1.11–1.37)	1.09 (0.94–1.26)
Average (AGA)	0.5		Reference
Large (LGA)	0.5	1.04 (0.93–1.15)	1.01 (0.88–1.17)
Ponderal index[c]			
Low ('thin')	0.6	1.11 (0.99–1.25)	1.12 (0.96–1.30)
Average	0.5		Reference
High ('plump')	0.5	1.02 (0.92–1.14)	1.06 (0.92–1.21)
Head circumference for gestational age			
Small	0.7	1.16 (1.05–1.28)	1.04 (0.90–1.19)
Average	0.5		Reference
Large	0.5	0.96 (0.85–1.07)	1.12 (0.97–1.29)
Birthweight (g)[d]			
<2,500	0.6	1.06 (0.90–1.25)	0.94 (0.73–1.21)
2,500–4,499	0.5		Reference
≥4,500	0.6	1.17 (0.94–1.44)	1.26 (0.96–1.66)

CI, confidence interval; SGA, small for gestational age; AGA, appropriate for gestational age; LGA, large for gestational age.

[a] Adjusted for all maternal characteristics (maternal age, parity, years of involuntary childlessness before index pregnancy, presence of diabetes or preeclampsia at index pregnancy, as well as for maternal educational level, country of birth, and PCOS diagnosis).

[b] Small and large birthweights for gestational age were defined as birthweights of more than 10% below or above, respectively, the mean birthweight for gestational age according to the Swedish sex-specific foetal growth curve (Marsal).

[c] Low and high ponderal indices were defined as a ponderal index score among the approximately 10% lowest or highest ponderal index in the study population.

[d] Small and large head circumferences for gestational age were defined as more than 10% below or above, respectively, the mean head circumference according to the Swedish reference curve for new-born infants (Niklasson).

Paper II

Women with PCOS were older ($p<0.01$), were more often primiparous ($p=0.01$), had more often conceived through ART ($p<0.01$) and more often developed preeclampsia ($p<0.01$) than women without PCOS. There was no

difference in BMI (the groups were matched by BMI), country of birth, smoking habits, infant size at birth, or other pregnancy complications between the groups.

Women with PCOS demonstrated higher AMH levels, total testosterone levels, and FAI during pregnancy than women without PCOS (all p-values <0.01) (Figure 2). Among women with PCOS, AMH levels were positively correlated with total testosterone levels (rho=0.17, p=0.031) and FAI (rho=0.16, p=0.045). In the control group, AMH levels were positively correlated with total testosterone levels (rho=0.26; p<0.001) and FAI (rho=0.23; p<0.001) (Figure 3). In women with PCOS, high testosterone levels (B=2.7, β=0.26, p<0.01) and low first-trimester BMI (B=-0.5, β=-0.17, p=0.04) remained independently associated with AMH levels, having taken PCOS phenotype, gestational length, age, and ART into account.

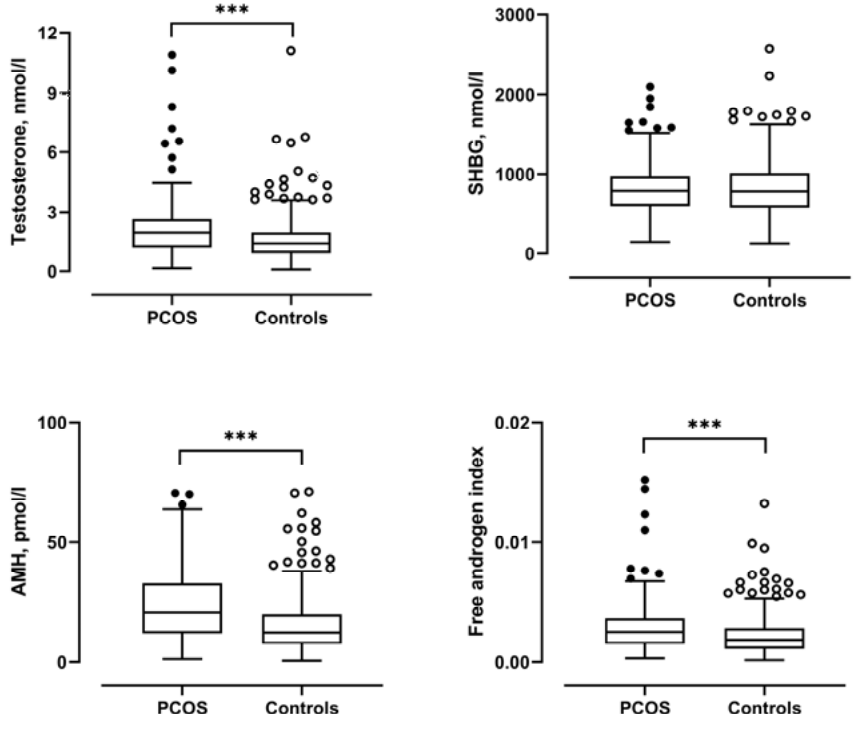

Figure 2. Early second-trimester hormone levels in women with PCOS (n=159) and controls (n=320); Total testosterone, sex hormone-binding globulin (SHBG), anti-Müllerian hormone (AMH) levels, and free androgen index. One high AMH value in the PCOS group has been omitted for clarity. Boxplots display median values, interquartile ranges and 5th and 95th percentiles. ***p<0.001.

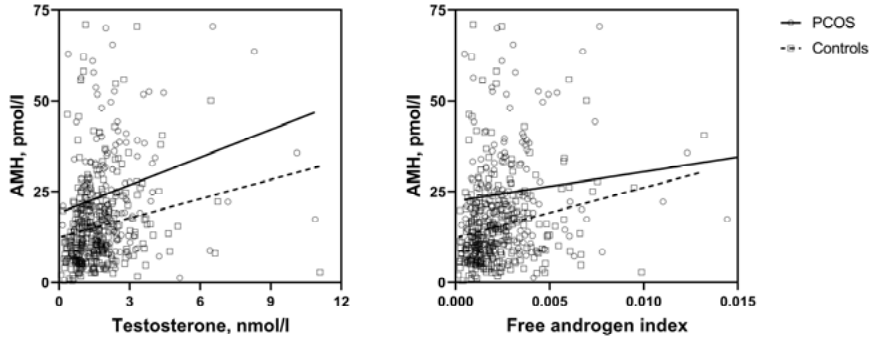

Figure 3. Correlations between anti-Müllerian hormone (AMH) and total testosterone and free androgen index in women with PCOS and controls. One high AMH value in the PCOS group has been omitted for clarity.

AMH levels were not associated with increased risk of adverse pregnancy outcomes or infant size at birth. AMH levels were negatively associated with risk for gestational hypertension (aOR=0.55, 95% CI 0.34–0.87) (Table 4).

Table 4. *Early second-trimester AMH levels and obstetric and neonatal outcomes in the entire study population.*

		AMH (pmol/l)	OR (95% CI)	*P*-value	aOR[a] (95% CI)	*P*-value
Gestational hypertension	No	15.1 (8.8–23.5)				
	Yes	12.8 (5.6–19.7)	0.57 (0.37–0.88)	0.011	0.55 (0.34–0.87)	0.012
Preeclampsia	No	14.8 (8.6–23.3)				
	Yes	17.3 (8.4–23.5)	0.94 (0.53–1.65)	0.818	1.01 (0.56–1.82)	NS
Gestational diabetes	No	14.9 (8.7–23.4)				
	Yes	9.2 (7.5–19.1)	0.86 (0.36–2.06)	0.731	1.05 (0.36–3.09)	NS
Preterm birth	No	14.8 (8.6–23.3)				
	Yes	14.7 (11.1–22.6)	1.11 (0.62–1.98)	0.723	1.20 (0.68–2.12)	NS
SGA	No	14.9 (8.6–19.9)				
	Yes	10.1 (7.4–13.4)	0.64 (0.25–1.64)	0.351	0.54 (0.18–1.64)	NS
LGA	No	14.8 (8.6–23.8)				
	Yes	15.2 (8.6–23.8)	1.00 (0.62–1.62)	0.988	1.16 (0.67–2.00)	NS

AMH levels displayed as medians (interquartile ranges). Statistical analyses based on multiple logistic regression. AMH was modelled as a continuous variable. NS, not significant; SGA, small for gestational age; LGA, large for gestational age.

[a] Adjusted for age, parity, maternal country of birth, BMI, smoking, gestational length at blood sampling, and year of delivery.

Paper III

In the final cohort of 1,046,448 women, 13,559 (1.3%) had a PCOS diagnosis before and/or during the index pregnancy. PCOS diagnoses became more frequent as the years passed; a total of 0.8% of the women giving birth in Sweden

during 2005–2008 had a PCOS diagnosis, while the corresponding rate was 1.9% during 2012–2014. Preterm birth was more common in women with PCOS (6.7%) than in women without PCOS (4.8%). When exploring severity of preterm birth, it was found that women with PCOS had increased risk of extremely (aOR 2.28, 95% CI 1.74–2.99), very (aOR 1.42, 95% CI 1.13–1.78), and moderately (aOR 1.28, 95% CI 1.18–1.39) preterm birth, compared with women without PCOS (Table 5).

Table 5. *Risk of preterm birth by severity and PCOS diagnosis, in women giving birth in Sweden 2005–2014.*

		Odds ratio (95% CI)		
	n (%)	Crude	Adjusted 1[a]	Adjusted 2[b]
Extremely preterm birth *(22–27 weeks)*				
No PCOS	2,346 (0.23)			Reference
PCOS	81 (0.60)	2.64 (2.11–3.30)	2.65 (2.04–3.44)	2.28 (1.74–2.99)
Very preterm birth *(28–31 weeks)*				
No PCOS	4,439 (0.43)			Reference
PCOS	93 (0.69)	1.61 (1.30–1.98)	1.55 (1.24–1.95)	1.42 (1.13–1.78)
Moderately preterm birth *(32–36 weeks)*				
No PCOS	42,375 (4.13)			Reference
PCOS	731 (5.46)	1.34 (1.24–1.45)	1.33 (1.23–1.45)	1.28 (1.18–1.39)

[a] Adjusted for maternal age, parity smoking habits, country of birth, and year of delivery.
[b] Adjusted for same covariates as Adjusted 1 and for BMI.

Among women with PCOS, 623 (4.6%) had spontaneous preterm births and 274 (2.0%) had medically indicated preterm births. The corresponding numbers in women without PCOS were 34,649 (3.4%) for spontaneous preterm birth and 13,896 (1.4%) for medically indicated preterm birth. Among women with PCOS who had spontaneous preterm births, 9.8% had extremely preterm births, 9.6% had very preterm births, and 80.6% had moderately preterm births. The corresponding proportions were 4.6%, 6.8%, and 88.6%, respectively, in women without PCOS. Among women with PCOS who had medically indicated preterm births, 6.9% had extremely preterm births, 10.9% had very preterm births, and 82.1% had moderately preterm births. The corresponding proportions were 4.8%, 14.2%, and 80.9%, respectively, in women without PCOS.

Maternal PCOS diagnosis was associated with increased risk of both spontaneous and medically indicated preterm birth (aOR=1.36, 95% CI 1.24–1.48 and aOR=1.31, 95% CI 1.15–1.50, respectively). For spontaneous preterm births, women with PCOS had increased risk for extremely (aOR 2.66, 95% CI 1.95–3.62), very (aOR 1.86, 95% CI 1.40–2.47), and moderately (aOR 1.26, 95% CI 1.14–1.38) preterm birth, compared with women without PCOS. For medically indicated preterm birth, women with PCOS had increased risk

for moderately preterm birth (aOR 1.36, 95% CI 1.17–1.57), but not for extremely (aOR 1.55, 95% CI 0.87–2.75) or very (aOR 0.96, 95% CI 0.65–1.42) preterm births (Table 6). In a restricted analysis, where all women with diabetes or hypertension diseases were excluded, the associations between PCOS and preterm birth were similar.

Table 6. *Risk of preterm birth in women with PCOS by mode of onset of delivery; preterm birth divided into extremely, very, and moderately preterm.*

		Odds ratio (95% CI)		
	n (%)	Crude	Adjusted 1[a]	Adjusted 2[b]
Spontaneous extremely preterm birth *(22–27 weeks)*				
No PCOS	1,585 (0.15)			Reference
PCOS	61 (0.45)	2.94 (2.26–3.84)	3.06 (2.27–4.11)	2.66 (1.95–3.62)
Spontaneous very preterm birth *(28–31 weeks)*				
No PCOS	2,362 (0.23)			Reference
PCOS	60 (0.44)	1.95 (1.51–2.52)	1.87 (1.42–2.48)	1.86 (1.40–2.47)
Spontaneous moderately preterm birth *(32–36 weeks)*				
No PCOS	30,702 (2.98)			Reference
PCOS	502 (3.72)	1.27 (1.16–1.40)	1.27 (1.16–1.39)	1.26 (1.14–1.38)
Medically indicated extremely preterm birth *(22–27 weeks)*				
No PCOS	669 (0.07)			Reference
PCOS	19 (0.14)	2.17 (1.38–3.43)	1.78 (1.01–3.16)	1.55 (0.87–2.75)
Medically indicated very preterm birth *(28–31 weeks)*				
No PCOS	1,980 (0.19)			Reference
PCOS	30 (0.22)	1.16 (0.81–1.67)	1.14 (0.78–1.68)	0.96 (0.65–1.42)
Medically indicated moderately preterm birth *(32–36 weeks)*				
No PCOS	11,247 (1.09)			Reference
PCOS	225 (1.67)	1.54 (1.35–1.76)	1.50 (1.30–1.73)	1.36 (1.17–1.57)

[a] Adjusted for maternal age, parity, smoking habits, country of birth, and year of delivery.
[b] Adjusted for same covariates as Adjusted 1 and for BMI.
Information on mode of onset of delivery was missing in 4,409 cases: 67 women with PCOS and 4,342 women without PCOS.

In a sensitivity analysis restricted to only primiparous women, the association between PCOS and extremely preterm birth was similar to that in the whole population (aOR 2.66 (95% CI 1.95–3.64).

Paper IV

In this study, the rate of stillbirth, expressed as 1/1,000, was 6.2 (n=260) in the PCOS group and 3.1 (n=745) in the non-PCOS group. Compared with non-PCOS women, women with PCOS had an association with 51% increased risk of stillbirth (aOR 1.51, 95% CI 1.28–1.78). When stratifying the PCOS group into women with and without anti-androgen medical treatment, an association was seen between PCOS women with no anti-androgen treatment

and increased risk of stillbirth, but no significant association between PCOS women with anti-androgen treatment and stillbirth in the adjusted analysis including BMI. Data are presented in Table 7.

Table 7. *Risk for stillbirth in women with PCOS giving birth in Sweden 1997–2007.*

	Stillbirth			
		Odds ratio (95% CI)		
	n (1/1,000)	Crude	Adjusted 1[a]	Adjusted 2[b]
No PCOS	745 (3.1)			Reference
PCOS	260 (6.2)	2.02 (1.76–2.33)	1.85 (1.61–2.14)	1.51 (1.28–1.78)
No anti-A treatment	193 (6.9)	2.23 (1.91–2.62)	1.99 (1.69–2.34)	1.60 (1.33–1.92)
Anti-A treatment	67 (4.9)	1.56 (1.24–2.04)	1.53 (1.19–1.97)	1.27 (0.96–1.68)

Anti-A treatment: Two dispensations of an anti-androgen drug during the years 2005–2017.
[a] Adjusted for maternal age, parity, type 1 diabetes, educational level, and country of birth.
[b] Adjusted for same covariates as Adjusted 1 and for BMI.

A chart was used to elucidate the rate of stillbirth across different gestational weeks in women with and without PCOS, see Figure 4. The chart shows that the rate of stillbirth becomes higher for PCOS women than non-PCOS women after 38 gestational weeks. The PAF for PCOS was 5.0 – that is, 5% of all stillbirths might be associated with PCOS.

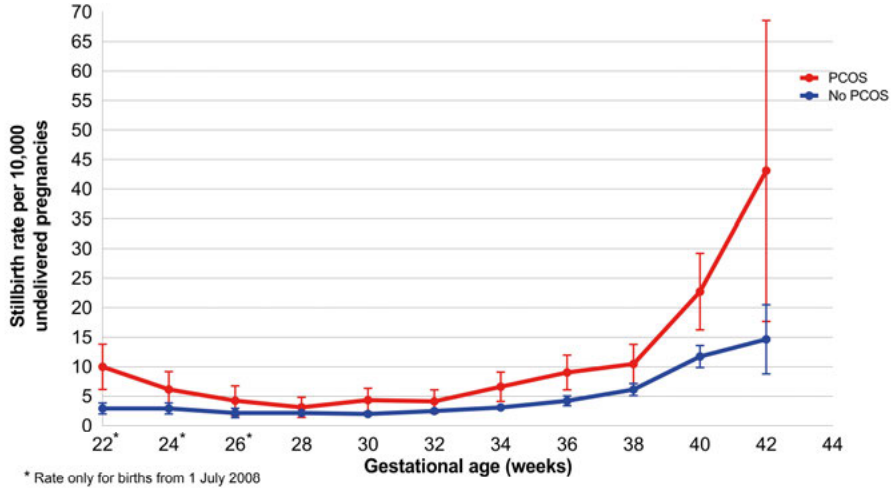

Figure 4. Rate of stillbirth by gestational age in Sweden years 1997–2015, stratified by PCOS diagnosis. Error bars indicate 95% confidence intervals.

Discussion

The studies in this thesis found that maternal obesity and smoking during pregnancy were associated with PCOS diagnosis in female offspring. Women with PCOS had a higher risk of spontaneous preterm birth of any severity than women without PCOS and the association was strongest with extremely preterm birth. However, preterm birth was not found to be associated with AMH levels in the matched cohort study. For medically indicated preterm birth, women with PCOS had increased risk of moderately preterm birth, but not extremely or very preterm birth. Women with PCOS had increased risk of stillbirth; the risk was not higher for PCOS women requiring anti-androgen medication than PCOS women without anti-androgen treatment.

Methodological considerations

Validity

When evaluating the validity of a study, both internal and external validities are to be considered. With high internal validity, the results are expected to be similar in repeated studies of the same population. External validity refers to if the study results can be applied to other populations, and external validity can never be high if internal validity is low.[154]

Three of the four studies were register-based. A register-based study design allows for a large study cohort. The information in these studies cannot be better than the information registered for the women. Cohort studies using MBR are of retrospective design, but the information in the MBR is prospectively collected during pregnancy and after birth, where neither the patient nor the caregiver are aware of future studies or can influence the data with these studies in mind, excluding the possibility of recall bias and decreasing information bias.

These three studies were nationwide, which makes it more likely that the results apply to women with PCOS in the whole of Sweden. They would probably also apply to countries with similar ethnicities in the population and similar socioeconomic status. On the other hand, Study II included only women in Uppsala county, diminishing the probability for the results to apply to all

PCOS women in Sweden. However, the results might apply to women with PCOS living in towns with similar age distribution, educational level, and socioeconomic status as Uppsala.

In Study III, the prevalence of PCOS was low – only 1.5%, but expected to be 5–10%. This can partly be explained by the fact that this was a fertile population (since all the women in the study were pregnant) and PCOS is associated with infertility. Another plausible explanation is that women with a PCOS diagnosis registered in the National Patient Register had severe symptoms, i.e., were inclined to seek medical care and be diagnosed with PCOS. Therefore, the study population probably consisted of women with more severe forms of PCOS, raising the question if the results would apply to all women with PCOS, or only women with severe forms of PCOS. The results might have been affected by this in at least two ways. If the association found between PCOS and preterm birth is true for women with all PCOS phenotypes, it is probably stronger than the results indicated, since the reference group included some women who should be in the PCOS group. If, on the other hand, the association is only true for the most severe cases of PCOS, the results probably showed a stronger association than would apply for all women with PCOS as one group. Regrettably, the register-based design of Study III did not allow for classification of different phenotypes.

In Study IV, an attempt was made to introduce a classification of women with PCOS with and without anti-androgen medical treatment. This was done in order to determine which group the possible association with stillbirth would apply to.

Bias (Systematic error)

Systematic errors are important factors that can affect associations found in epidemiological studies. Some of these factors can be minimised through careful study design, others can be adjusted for in the analyses, and still others are hard to affect but must be recognised.

Confounding

Confounding is one type of systematic error. A confounder is a factor that is a progenitor of both the exposure and the outcome. Another factor to be taken into consideration is a mediator, which comes between the exposure and the outcome in the causal pathway. For an explanation, see Figure 5.

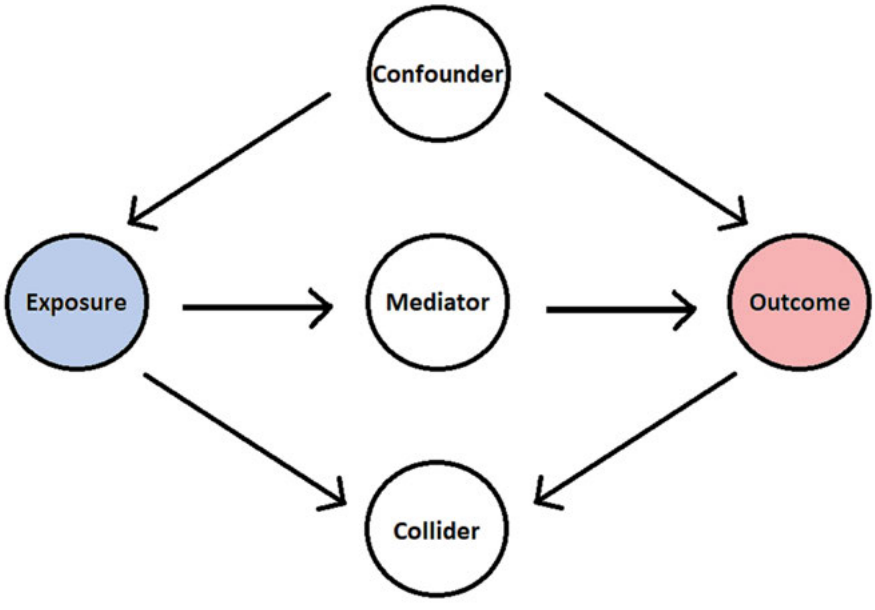

Figure 5. The relationships of confounders, mediators, and colliders to exposure and outcome.

For a systematic overview of possible causal pathways in Studies III and IV, DAGs were constructed[152] using the DAGitty web application from *www.dagitty.net*. The DAGs can be used to identify possible confounders, mediators, and colliders. When creating a DAG, the arrows from one factor to another can go in only one direction and cannot go in a cycle. In analyses of associations, non-causal pathways must be blocked, which is done by adjusting for confounders. Mediators are a part of the causal pathway and should not be adjusted for, since the pathway from the exposure to the outcome would then be blocked.

In these studies, my coresearchers and I were uncertain if BMI should be seen as a confounder or a mediator in the causal pathway from PCOS to pregnancy complications. Overweight and obesity might increase the risk of symptoms of PCOS, but overweight and obesity can also be consequences of PCOS. Because of this uncertainty, we decided to do the analyses in two steps, first without adjusting for BMI, and then with adjustment for BMI.

Selection bias

Selection bias is a systematic error that is introduced into a study, for example when recruiting study participants.[154] In Study II, the women who chose to participate in the study might have had a higher educational level[155] and higher socioeconomic status than women not participating, since they would be more aware of the importance of scientific research and the inclusion of participants

to perform studies. Another bias can be that women with a problem or disease during pregnancy are more aware and willing to participate in a study. Women were also required to be able to speak Swedish in Study II, which excludes women recently immigrated from other countries. In Studies I, III, and IV, the selection bias was diminished since the studies were nation-wide. All pregnancies in Sweden during the study period were included in Studies I and III and pregnancies were randomly selected from the whole country in Study IV. The design using the MBR (Studies I, III, and IV), to which all births in Sweden must be reported, decreases selection bias.

Information bias

Information biases include, for example, classification errors or measurement errors.[154] Classification errors can occur for example when grouping people in a study by exposure and result in skewed estimates of the association between exposure and outcome. Measurement error can occur for example if measurements are wrongly estimated or documented. Studies I, III, and IV were register-based, which means that classification bias may have been introduced.

In Study I, the female offspring could only be followed up to a maximum age of 28 years, with a risk of females who got their PCOS diagnosed later in life being classified as a non-PCOS outcome. This would introduce a classification bias, expected to weaken the observed association. In Studies I and III, the prevalence of PCOS was lower than expected. This leads to suspicion of misclassification bias; it can be expected that there are women with PCOS who had not yet sought medical care and been given the diagnosis, in the non-PCOS group. Thus, women in the National Patient Register with a PCOS diagnosis might be a group with a more severe form of PCOS, while women with a milder form of PCOS have not sought medical care and received a diagnosis. This misclassification would not be assumed to weaken the association found between PCOS and preterm birth. Since the group with women without PCOS diagnosis is very large, this effect on the estimates should be minimal.

An important limitation in Study II, which was a matched cohort study, is that PCOS cases were identified from a hospital register and a substantial number of women received their diagnosis as part of an infertility work-up and treatment where the clinical evaluation did not include full assessment of hyperandrogenism. Thus, the hyperandrogenic phenotype is probably underreported in the PCOS sample. Misclassification may have occurred in the control group, and in attempt to reduce this risk, controls with high AMH levels were specifically checked for clinical signs of undiagnosed PCOS.

In Study IV, women were included in the PCOS group regardless of whether they had the PCOS diagnosis registered in the National Patient Register before

or after the index pregnancy. The justification for this was that women with PCOS were considered likely to have had the underlying changes in pathophysiology already many years before diagnosis, even if they did not seek for symptoms until later on. They can even have had problems or already sought for symptoms such as irregular menstruations, acne, or hirsutism during adolescence or early adult life, without the physician paying attention to further PCOS symptoms, hence not making the PCOS diagnosis at that point. The pathophysiological factors related to PCOS, such as insulin resistance, hyperandrogenism, and chronic inflammation may therefore have influenced their pregnancies prior to the confirmed diagnosis. If these PCOS women did not have any traits of PCOS during the index pregnancy, the expected effect would be to weaken the observed association between PCOS and stillbirth. In Study IV, the researchers also identified the PCOS women with the most severe hyperandrogenic symptoms and thereafter stratified PCOS women into two categories: those who had received anti-androgenic drug treatment and those who had not. This is certainly not excluded from the risk of classification bias. Also, the group of women receiving anti-androgen drugs might have been heterogenic in regard to the indication for the drug prescription. Some of the PCOS women receiving anti-androgen combined oral contraceptives can have received the pills primarily as birth control, not as medication for hyperandrogenism. Further, since the Drug Prescription Register was not established until 2005, some of the older women in the study might have gotten prescriptions of anti-androgenic drugs only before 2005 and will therefore wrongly be classified as not having received anti-androgenic drug treatment. It must also be recognised that some women with hyperandrogenic symptoms needing treatment, receive treatment other than anti-androgenic drugs, such as laser treatment or waxing for hirsutism and antibiotics or retinoic acid treatment for acne. Further, some women with severe hyperandrogenic symptoms never seek care to get treatment. The effect of the classification biases mentioned would be expected to attenuate a possible difference in the association of stillbirth between the treatment and no treatment groups of PCOS women.

The register-based setup of the studies made it possible to sample information on many important covariates, such as BMI, smoking, infertility factors, socioeconomic factors, country of birth, and year of delivery. Information on covariates was, in all the studies, collected prospectively in a standardised manner by health care professionals, which reduces recall bias. Maternal weight was measured in a standardised manner with the woman wearing light indoor clothing, minimising informational bias. The validity of gestational age data in the MBR has been evaluated as acceptable, with a low error rate.[139] Information on BMI was missing for one third of the women in Paper I. However, when a stratified analysis was performed on women with and without information about BMI, the results were similar in both groups, indicating that this did not influence the findings. It is even debatable if BMI should be considered

as a mediator or confounder (in Papers III and IV). High BMI can be part of the PCOS status, but increasing BMI is also associated with higher risk of presenting with PCOS and could thus be considered a confounder.

Random error

Random error is an error that occurs in a study and is not systematic.[154] In studies, random errors can be introduced in the exposure, the outcome, and other covariates collected. The larger the study cohort, the lower the risk for random error in the data collection. The large cohort size in Studies I, III, and IV was an important strength, where the random error was expected to be minimal. In Study II, where the PCOS women were only 159, more risk of random error was introduced, for example in regard to basic characteristics in the cohort, the frequency of different phenotypes in the PCOS women, as well as the frequency of pregnancy complications in the group.

Data on BMI was missing for 38% of the mothers in Study I and for 7.8% of the mothers in Study III. In Study I, this was partly because information on weight was not registered in the MBR during certain years, thus introducing random error. Still, information on BMI was also missing for women giving birth during other periods in Study I as well as in Study III, raising speculations about if women with missing information to a greater extent were overweight and obese, and were not weighed either because of discomfort with drawing attention to weight on the part of the health care personnel, or because of the women themselves refusing to be weighed. Pregnancies with missing information on BMI were excluded in the statistical models including BMI. Another possibility would be to impute values for missing BMI, but that could yield skewed results, in accordance with the theory on non-random missing information on BMI.

General discussion

The associations found in this thesis can either be an estimate of a direct effect of the exposure on the outcome, or a proxy for another biological causal pathway. Which one of these hypotheses is correct cannot be answered in this thesis, but the findings certainly raise questions on possible pathophysiological implications. Even associations that are not a direct effect are important to observe, since they can give an opportunity to identify persons at risk for certain outcomes.

The association found between maternal overweight and obesity and later development of PCOS in offspring indicates that high BMI can be an independent risk factor. This could be a consequence of genetic factors – perhaps some

of the mothers with high BMI had PCOS despite not being aware of the diagnosis, which could contribute to explaining the significant association despite adjustment for maternal PCOS diagnosis. Another explanation could be that maternal abundant fat tissue might have direct or indirect effect on the foetus or the placenta. This could be through synthesis and secretion of adipocytokines, altered inflammatory factor profiles, or disturbed glucose metabolism with altered hormonal profile. Reduced mitochondrial biogenesis has been noted in the placenta of overweight or obese women, as have mitochondrial dysfunction and decreased placental levels of adenosine triphosphate.[156] Obesity can also interfere with the initial steps of cholesterol metabolism in the placenta, which can in turn lead to lower synthesis of oestradiol and progesterone.[157] Further, with increasing BMI, higher serum levels of maternal testosterone are seen during pregnancy,[37] and higher GWG is associated with higher levels of amniotic fluid testosterone levels in pregnancies with female foetuses.[38] Maternal obesity might thus affect the foetal hormonal profile and influence development of PCOS in offspring. A further explanation, but with weaker coupling to pathophysiology, might be that daughters have similar lifestyle habits as their mothers, as regards both exercise and nutrition. This might result in increased risk of daughters to mothers with high BMI themselves become overweight and obese, and thereby increasing their risk of developing PCOS.

The studies included herein have also confirmed association between maternal smoking and PCOS diagnosis in offspring; the strength of the association was dose-dependent. This association had not been observed earlier, to my knowledge, nor has it been inspected in further studies after the publication of Paper I. The association remained significant after adjustment for maternal BMI, educational level, and PCOS diagnosis. Maternal smoking during pregnancy has in the past been associated with decreased levels of reproductive hormones[158] and reduced fertility[159] in female offspring. Maternal smoking might also have direct effect on the foetal gonads. Foetal ovaries exposed to maternal smoking have a higher density of primordial follicles,[39] and reduced proliferation of germ cells and somatic cells.[40, 41, 160] It has even been linked to dysregulation of genes that are important for ovarian development.[39]

Relative birthweight was associated with later development of PCOS only in crude estimates, not after adjustments. Studies of this are not consistent and have demonstrated that both low and high birthweight can be associated with a later PCOS diagnosis.[16, 32, 33] According to Melo et al., a higher prevalence of PCOS was found in women who were born SGA than in women born AGA.[33] On the contrary, Davies et al. did not demonstrate higher prevalence of PCOS in women who were born SGA or women born LGA, but did find that a low ponderal index was associated with the diagnosis of PCOS where women had all the three diagnostic criteria for PCOS.[32] Further, Mumm et al.

found, in a large cohort study, that high birthweight, but not SGA or LGA, was associated with later development of PCOS.[16] Study I could not confirm association between actual birthweight and later PCOS, which is in agreement with several other studies.[34, 36, 161, 162] Interestingly, an association was found between both SGA and small head circumference for gestational age and later PCOS in the crude analysis. This connection found between relative size at birth and later development of PCOS underlines the importance of prenatal factors for the fully expressed syndrome. However, the association was attenuated and no longer significant after adjustment for maternal characteristics in the index pregnancy, indicating that maternal factors might be an underlying cause of the association of relative size at birth and later PCOS.

The findings that second-trimester AMH levels in pregnant women with PCOS were higher than in controls were in line with previous studies.[42, 163] The AMH levels were positively correlated with total testosterone levels and with FAI, in both women with and women without PCOS. This is in agreement with a study in mice that has confirmed higher testosterone levels in both AMH-exposed dams and their offspring.[42] The same study showed phenotypic traits of PCOS in the offspring of AMH-exposed dams, but offspring birthweight was unaffected.[42] This is also in line with Study II, which did not show an association of maternal AMH levels with offspring birthweight or the risk of SGA or LGA. The fact that AMH and testosterone levels are positively correlated in pregnancy suggests that the ovaries are actively producing testosterone during pregnancy, at least until the early second trimester. In the placenta, androgens are metabolised to oestrogens by aromatase,[164] but high AMH levels might also affect testosterone production in the placenta,[42] contributing to their relationship.

Study II did not find that higher AMH levels were associated with increased risk of PCOS-related pregnancy complications. On the contrary, a decreased risk for gestational hypertension was observed with higher AMH levels, which is in accordance with previous studies.[66, 67] A possible explanation to this could be that hypertensive disorders in pregnancy indicate impaired vascular health, and lower levels of AMH reflect impaired ovarian blood supply.[165] While the literature on AMH and hypertensive disorders in pregnancy is inconclusive,[68] none of the previous studies included women with PCOS.

Several factors can be involved in the mechanisms increasing the risk of preterm birth and stillbirth in PCOS women, as observed in this thesis, such as overweight and obesity, concurrent diseases during pregnancy, and chronic inflammation with oxidative stress. Women with PCOS had increased risk of extremely preterm birth, with the strongest association to spontaneous onset. Women with PCOS have chronic systemic low grade inflammation, probably largely related to characteristics in their adipose tissue,[115, 116] and this could

contribute to their increased risk of spontaneous preterm birth.[96, 117] The risk of late miscarriage and preterm delivery in women with PCOS appears, in pooled data, to be reduced through metformin treatment, and this effect is seen for both hyper- and normoandrogenic women.[166] This is probably the result of the anti-inflammatory effects of metformin.[167] Hyperandrogenism might also affect the risk of spontaneous preterm birth in women with PCOS,[81] but the risk of preterm birth by PCOS phenotype could not be inspected in Study III because of the study design.

The results also indicated that women with PCOS had increased risk of medically indicated moderately preterm birth. This can be mediated though concurrent diseases, such as hypertonic and diabetic diseases. Still, the association was significant even when women with these diseases were excluded from the analyses, suggesting that other factors related to PCOS also impact on this association.

Earlier studies have not shown any association of PCOS and the risk of stillbirth, as seen in Study IV. Overweight and obesity, which are more common in women with PCOS, are associated with stillbirth.[168] Further, gestational hypertension and preeclampsia are associated with similar placental lesions as are found in stillbirth,[169] and those diseases are more frequent in women with PCOS. A possible mechanism linking PCOS to stillbirth is an effect on placentation with subsequent foetal growth restriction, as stillbirth is strongly associated with foetal growth restriction[170]. Studies on a possible association between PCOS and foetal growth restriction are scarce. One study has shown such association, but the rate of duplex pregnancies in that study was considerably larger in the PCOS group compared with in the non-PCOS group.[171] Other studies, that have not confirmed this association, had small study populations[172] or no stated definition of foetal growth restriction,[173-175] making it difficult to interpret the results. Women with PCOS have increased risk of children born SGA,[84, 176] where SGA children either are constitutionally small or the small size for gestational age is a result of foetal growth restriction.[130] Further, foetal growth restriction has been linked to oxidative stress,[177] and women with PCOS more frequently present with oxidative stress, chronic inflammation, and mitochondrial dysfunction.[178] In addition, in recent studies on rats, foetal loss in PCOS-like dams was associated with oxidative stress caused by increased production of reactive oxygen species and inactivation of antioxidative proteins in the uterus and the placenta.[179, 180] Ferroptosis is also a potential mechanism leading to uterine and placental dysfunction in PCOS-like dams, subsequently leading to foetal loss.[181]

Clinical implications and future perspectives

This thesis emphasises the importance of good information to women about general health. Normal weight in fertile age is something to strive for, since overweight or obesity not only negatively affects a pregnant woman, but can also increase the risk of diseases such as PCOS later in the life of her offspring. Further, the results underline the importance of non-smoking during pregnancy to decrease the risk of possible later development of PCOS in female offspring.

In current practices in maternal care in Sweden, and probably worldwide, PCOS is not regarded as a risk factor for pregnancy complications and no special attention is paid to women with PCOS. However, women with PCOS should, at diagnosis, be informed about their increased risk of pregnancy complications. This could encourage healthy lifestyle and maintenance of normal weight before conceiving and reduce the risk of excessive weight gain during pregnancy, which can otherwise further increase the risk for complications.

It is also important to raise the question of how to monitor women with PCOS in maternal care, and if, in fact, they should be monitored more closely than women without PCOS. Closer monitoring of cervix length would be one possibility to identify women with a shortened cervix, and therefore increased risk for preterm birth. Still, the probability to capture the risk of preterm birth in women with PCOS through cervix measurements would first require further investigation. In women with identified increased risk of preterm birth, treatment with metformin or vaginal progesterone might be considered. PCOS could also be considered as one of several factors in a predictive model for preterm births. Further studies are warranted before this would be a general recommendation.

Another factor to pay attention to is the higher rate of stillbirths after 38 gestational weeks among women with PCOS compared with women without PCOS. Routine foetal growth scans and umbilical artery Doppler ultrasound in women with PCOS close to estimated birth could be applied to discover foetuses with growth restriction or impaired placental function. Subsequently, birth could be induced in women with abnormal results. As with preterm birth, the association of stillbirth in women with PCOS needs to be further examined before any such recommendations would become relevant in clinical settings.

Given current evidence, measurement of AMH levels, expressing ovarian morphology, cannot be recommended for routine use in maternal care to predict the risk of pregnancy complications related to PCOS, such as gestational diabetes, gestational hypertension and preeclampsia, and preterm birth.

Conclusions

Factors during intrauterine life, such as maternal smoking and obesity, may influence the risk for later PCOS in offspring. Hopefully, it will be possible to restrict maternal smoking or obesity with education and interventions in primary maternal care or before the start of planning a family. Being born SGA was associated with PCOS development, but the association seemed to be mediated by maternal factors. Possible maternal factors included steroid hormones and the results of Paper II indicated that AMH levels were higher in women with PCOS in the second trimester of pregnancy, and that high testosterone and low BMI were the main predictors of pregnancy AMH levels in women with PCOS. High AMH levels are not *per se* associated with increased risk for PCOS-associated maternal or neonatal complications such as preterm birth. Thus, high levels of AMH should not be considered to be a primary factor in the generally increased risk for adverse obstetric outcomes in women with PCOS.

Women with PCOS diagnosis were at increased risk of both extremely preterm birth and stillbirth compared with women without PCOS. For spontaneous onset of extremely preterm delivery, the risk was increased by threefold. Though extremely preterm births are not very common, they can result in severe morbidity and mortality of the infant, causing a great burden to the individual and the family, as well as a great cost to society. Stillbirths are also rare, but are a devastating occurrence for the mother, as well as the child's whole family. This underlines the importance of identifying PCOS as an important risk factor for pregnancy complications, including severe ones.

Summary in Swedish – Sammanfattning på svenska

Polycystiskt ovarialsyndrom (PCOS) är en av de vanligaste metabola störningarna och drabbar runt 10–15% av kvinnor i fertil ålder. Enligt Rotterdam kriterierna som introducerades år 2003, behöver kvinnor uppfylla åtminstone 2 av följande 3 kriterier för att få diagnosen PCOS: 1) Oligo- eller anovulation, 2) Hyperandrogenism, antingen klinisk i form av hirsutism eller svår acne, eller biokemisk med förhöjt testosteron och 3) Polycystiska äggstockar vid ultraljudsundersökning. Det är ofullständigt känt varför vissa kvinnor drabbas av PCOS och andra inte. Förmodligen är det ett komplext samspel mellan ärftlighet och miljö, och eventuellt kan miljön redan i fosterlivet ha betydelse. Kvinnor med PCOS har ökad risk för infertilitet, insulinresistens, typ 2-diabetes och hjärt- och kärlsjukdomar senare i livet. Under graviditet har PCOS-kvinnor ökad risk för komplikationer så som missfall, graviditetsdiabetes, barn födda små eller stora för tiden, förtidsbörd, graviditetshypertoni och havandeskapsförgiftning. Kvinnor med PCOS har ofta förhöjt anti-Müllerianhormon (AMH), som är producerat i preantral- och små antralfolliklar i äggstockarna. Det är oklart om de förhöjda AMH-nivåerna bidrar till PCOS-kvinnornas förhöjda risk för graviditetskomplikationer. Syftet med denna avhandling var att få ökad kunskap om associationen mellan intrauterin miljö och risk att utveckla PCOS samt att ytterligare undersöka associationen mellan PCOS och graviditetskomplikationer.

I första delarbetet undersöktes om faktorer hos mamman under graviditet eller flickans storlek vid födseln påverkar risk för att utveckla PCOS senare i livet. Denna registerbaserade studie innefattade alla enkelbörder med flickor i Sverige år 1982–1995. Utfall var PCOS-diagnos hos flickorna från 15 års ålder och vi hade möjlighet att följa dem upp till högst 28 års ålder. Studien visade att om mamman var överviktig/hade fetma eller rökte under graviditet, ökade risken för PCOS hos dottern senare i livet jämfört med flickor som hade normalviktiga mammor eller mammor som inte rökte. Storlek vid födseln var inte associerad med PCOS.

I andra delarbetet undersöktes om AMH-nivåer under tidig andra trimester var högre hos PCOS-kvinnor än icke-PCOS-kvinnor, om AMH-nivåerna var korrelerade med testosteron-nivåer, och om höga AMH-nivåer var associerade

med ökad risk för graviditetskomplikationer. Detta var en matchad kohortstudie, som innefattade 479 gravida kvinnor, varav 159 kvinnor med PCOS och 320 kontroller med enkelbörd i Uppsala år 2007–2015. Kvinnor med och utan PCOS var matchade för BMI och kalendervecka vid blodprovstagning. Information om PCOS-diagnos samt graviditetskomplikationer inhämtades från patientjournaler och blodprover togs i samband med rutinultraljudsundersökningen i andra trimestern. Kvinnor med PCOS hade högre nivåer av AMH under graviditet än kontroller, och AMH-nivåer under andra trimestern var korrelerade med testosteron-nivåer. Höga AMH-nivåer var inte associerade med ökad risk för PCOS-relaterade graviditetskomplikationer eller risk för att föda barn som var för litet eller stort för tiden. Däremot sågs en lätt sänkt risk för graviditetshypertoni.

I tredje delarbetet undersöktes om PCOS var associerat med förtidsbörd av olika svårighetsgrader, det vill säga om barnet var fött extremt (<28 graviditetsveckor), mycket (28–31 graviditetsveckor) eller måttligt (32–36 graviditetsveckor) prematurt. Ytterligare undersöktes om PCOS-relaterad förtidsbörd hade spontan eller iatrogen start. Studien var registerbaserad och innefattade alla enkelbörder i Sverige år 2005–2014. Kvinnor med PCOS hade ökad risk för förtidsbörd av alla svårighetsgrader jämfört med kvinnor utan PCOS-diagnos. Associationen var starkast med extrem förtidsbörd med spontan förlossningsstart; där den justerade risken var 2,5 gånger högre än hos kvinnor utan PCOS.

I fjärde delarbetet undersöktes om kvinnor med PCOS hade ökad risk för intrauterin fosterdöd. Studien var en registerbaserad kohortstudie och den innefattade 283 433 enkelbörder förlösta år 1997–2015, varav 41 827 av en kvinna med PCOS och 241 606 av en kvinna utan PCOS. Kvinnor med PCOS delades upp i två grupper baserat på om de under åren 2005–2015 medicinerat med antiandrogena mediciner (minst två receptuttag) eller ej. Kvinnor med PCOS hade 1,5 gånger ökad risk för intrauterin fosterdöd jämfört med kvinnor utan PCOS-diagnos. Det var framför allt vid och efter fullgången tid som frekvensen av intrauterin fosterdöd var högre hos PCOS-kvinnorna. PCOS-kvinnor som medicinerat med antiandrogena mediciner hade inte starkare association med intrauterin fosterdöd än PCOS-kvinnor som inte hade det.

Sammanfattningsvis så ökar högt BMI och rökning under graviditet risken för att dottern senare i livet ska få en PCOS-diagnos. AMH-nivåer under graviditet är inte associerade med ökad risk för PCOS-relaterade graviditetskomplikationer. Kvinnor med PCOS har ökad risk för allvarliga graviditetskomplikationer så som extrem förtidsbörd och intrauterin fosterdöd jämfört med kvinnor utan PCOS.

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