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DEVELOPMENTAL ORIGINS OF POLYCYSTIC OVARY SYNDROME: ROLE OF EARLY ADVERSE LIFE EVENTS ON ADULT HEALTH

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Developmental origins of polycystic ovary syndrome: Role of early adverse life events on adult health

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To my beloved family

Στην οικογένειά μου

ABSTRACT

The research focus of this thesis is polycystic ovary syndrome (PCOS), the most common endocrine disorder among women of reproductive age, associated with reproductive, cardio-metabolic, and mental health complications. Despite the high prevalence, little is known about the etiology of the syndrome. The scope of the current thesis was to investigate aspects of the developmental origins of the syndrome, with focus on the impact of adverse environmental factors during pregnancy on PCOS-associated features in the offspring. To reach these aims we employed three mouse model of PCOS and data from a Swedish national register-based cohort study.

The thesis is divided into two parts. **Part 1** investigates the effects of maternal androgen excess and maternal obesity on the mental health of adult male and female offspring. **Study I** demonstrated that maternal androgen exposure increases anxiety-like behavior in the first generation of female mouse offspring, but not male offspring, while maternal obesity affects male offspring behavior, but not female offspring. This sexually dimorphic response to the two prominent environmental stimuli was further supported by sex-specific changes in gene expression within the amygdala and hypothalamus. **Study II** provided evidence that daughters of women with PCOS are at increased risk to be diagnosed with anxiety disorders, but not the sons, using a Swedish national register-based cohort study. It further showed that maternal androgen exposure can lead to transgenerational transmission of anxiety-like behavior in the third generation of female mice, but not the male offspring, without major impact of maternal obesity. Finally, the male germline (first generation of male offspring with no change in behavior) did also transgenerationally transmit an anxiety-like behavior to the third generation of male offspring.

Part 2 investigates the effects of an adverse maternal and/or postnatal environment on the cardiovascular and metabolic health of females, using mouse models of PCOS. **Study III** revealed that maternal androgen excess leads to cardiac hypertrophy in adult female offspring, accompanied by gene expression changes in the left ventricle, without the presence of metabolic dysfunction. It was further shown that this adverse cardiac phenotype is the result of an early cardiac reprogramming. In addition, cardiovascular assessment of a mouse model of PCOS with continuous exposure to androgens from prepuberty to adulthood, demonstrated cardiac hypertrophy and increased vasocontractile responses, while simultaneous administration of the anti-androgen flutamide could only partially prevent the observed phenotype. Finally, in **study IV**, developmental, reproductive and metabolic complications were revealed in a transgenic mouse model of PCOS that overexpresses ovarian nerve growth factor. Ovarian excess of nerve growth factor led to developmental defects in the female fetus, including growth restriction, reduction of germ cell number and delayed primary oocyte maturation. In addition, the adult transgenic female mice displayed disrupted estrous cyclicity and ovarian expression changes of steroidogenic genes and epigenetic markers. Lastly, these mice developed metabolic complications, as shown by impaired glucose metabolism, aberrant adipose tissue function, and liver steatosis.

LIST OF SCIENTIFIC PAPERS

I. **Manti M**, Fornes R, Qi X, Folmerz E, Linden Hirschberg A, de Castro Barbosa T, Maliqueo M, Benrick A, Stener-Victorin E. (2018) Maternal androgen excess and obesity induce sexually dimorphic anxiety-like behavior in the offspring. FASEB J 32, 4158-4171

II. Risal S, **Manti M**, Lu H, Fornes R, Larsson H, Benrick A, Deng Q, Cesta CE, Rosenqvist MA, Stener-Victorin E. Prenatal androgen exposure causes a sexually dimorphic transgenerational increase in offspring susceptibility to anxiety disorders. (manuscript)

III. **Manti M**, Fornes R, Pironti G, McCann Haworth S, Zhengbing Z, Benrick A, Carlström M, Andersson D, Stener-Victorin E. (2019) Maternal androgen excess induces cardiac hypertrophy and left ventricular dysfunction in female mice offspring. Cardiovasc Res. pii: cvz180. doi: 10.1093/cvr/cvz180

IV. **Manti M***, Pui HP*, Edström S, Risal S, Lu H, Lindgren E, Jerlhag E, Benrick A, Deng Q, Stener-Victorin E. Excess of ovarian nerve growth factor impairs embryonic development and causes reproductive and metabolic dysfunction in adult mice. (manuscript)

* Equal Contribution

ADDITIONAL SCIENTIFIC PAPERS NOT INCLUDED IN THIS THESIS

I.   Risal S, Pei Y, Lu H, **Manti M**, Fornes R, Pui HP, Zhao Z, Massart J, Ohlsson C, Lindgren E, Crisosto N, Maliqueo M, Echiburú B, Ladrón de Guevara A, Sir-Petermann T, Larsson H, Rosenqvist MA, Cesta CE, Benrick A, Deng Q, Stener-Victorin E. (2019) Prenatal androgen exposure and transgenerational susceptibility to polycystic ovary syndrome. Nat Med. 25(12):1894-1904. doi: 10.1038/s41591-019-0666-1

II. Stener-Victorin E, **Manti M**, Fornes R, Risal S, Lu H, Benrick A. (2019) Origins and Impact of Psychological Traits in Polycystic Ovary Syndrome. Med Sci (Basel). pii: E86. doi: 10.3390/medsci7080086

III. Fornes R, **Manti M**, Qi X, Vorontsov E, Sihlbom C, Nyström J, Jerlhag E, Maliqueo M, Hirschberg AL, Carlström M, Benrick A, Stener-Victorin E. (2019) Mice exposed to maternal androgen excess and diet-induced obesity have altered phosphorylation of catechol-O-methyltransferase in the placenta and fetal liver. Int J Obes (Lond). 43(11):2176-2188. doi: 10.1038/s41366-018-0314-8.

IV. Lindheim L, **Manti M**, Fornes R, Bashir M, Czarnewski P, Diaz OE, Seifert M, Engstrand L, Villablanca EJ, Obermayer-Pietsch B, Stener-Victorin E. (2018) Reproductive and Behavior Dysfunction Induced by Maternal Androgen Exposure and Obesity Is Likely Not Gut Microbiome-Mediated. J Endocr Soc. 2(12):1363-1380. doi: 10.1210/js.2018-00266.

CONTENTS

LIST OF ABBREVIATIONS

PCOS	Polycystic ovary syndrome
NIH	National Institutes of Health
AE-PCOS	Androgen-Excess PCOS society
LH	Luteinizing hormone
FSH	Follicle stimulating hormone
LHCGR	Luteinizing Hormone/Choriogonadotropin Receptor
FSHR	Follicle Stimulating Hormone Receptor
THADA	THADA Armadillo Repeat Containing
DENND1A	DENN Domain Containing 1A
FSHB	Follicle Stimulating Hormone Subunit Beta
RAB5B/SUOX	RAB5B, Member RAS Oncogene Family/ Sulfite Oxidase
HMGA2	High Mobility Group AT-Hook 2
TOX3	TOX High Mobility Group Box Family Member 3
INSR	Insulin Receptor
BMI	Body mass index
PNA	Prenatally androgenized
CD	Control diet
HFHS	High-fat high-sucrose
VEH	Vehicle
DHT	Dihydrotestosterone
F1-F2-F3	First-second-third generation
AGD	Anogenital distance
NGF	Nerve growth factor
GD	Gestational day

Genes tested in the mouse models presented in the thesis

Gene	Name	Gene	Name
Adora2a	Adenosine A2a Receptor	*Il1r1*	Interleukin 1 Receptor Type 1
Adra1a	Adrenoceptor Alpha 1A	*Insr*	Insulin Receptor
Adra1b	Adrenoceptor Alpha 1B	*Irs1*	Insulin Receptor Substrate 1
Adrb3	Adrenoceptor Beta 3	*Lepr*	Leptin Receptor
Ar	Androgen receptor	*Maoa*	Monoamine Oxidase A
Atp2a2	ATPase Sarcoplasmic/Endoplasmic Reticulum Ca2+ Transporting 2	*Mef2c*	Myocyte Enhancer Factor 2C
Bdnf	Brain Derived Neurotrophic Factor	*Ngfr*	Nerve Growth Factor Receptor
Btg2	BTG Anti-Proliferation Factor 2	*Nox4*	NADPH Oxidase 4
Cacna2d1	Calcium Voltage-Gated Channel Auxiliary Subunit Alpha2delta 1	*Nppb*	Natriuretic Peptide B
Camk2n1	Calcium/Calmodulin Dependent Protein Kinase II Inhibitor 1	*Nr3c1*	Nuclear Receptor Subfamily 3 Group C Member 1
Cebpbeta	CCAAT Enhancer Binding Protein Beta	*Nrg1*	Neuregulin 1
Col1a1	Collagen Type I Alpha 1 Chain	*Ntrk1*	Neurotrophic Receptor Tyrosine Kinase 1
Col6a1	Collagen Type VI Alpha 1 Chain	*Ntrk2*	Neurotrophic Receptor Tyrosine Kinase 2
Col6a3	Collagen Type VI Alpha 3 Chain	*Odc1*	Ornithine Decarboxylase 1
Comt	Catechol-O-Methyltransferase	*Otx1*	Orthodenticle Homeobox 1
Crebbp	CREB Binding Protein	*Ppardelta*	Peroxisome Proliferator Activated Receptor Delta
Crh	Corticotropin Releasing Hormone	*Ppargamma*	Peroxisome Proliferator Activated Receptor Gamma
Crhr1	Corticotropin Releasing Hormone Receptor 1	*Ryr2*	Ryanodine Receptor 2
Crhr2	Corticotropin Releasing Hormone Receptor 2	*Sirt1*	Sirtuin 1
Dbh	Dopamine Beta-Hydroxylase	*Slc17a7*	Solute Carrier Family 17 Member 7
Ep300	E1A Binding Protein P300	*Slc2a4*	Solute Carrier Family 2 Member 4
Erbb4	Erb-B2 Receptor Tyrosine Kinase 4	*Slc6a4*	Solute Carrier Family 6 Member 4
Ers1	Estrogen Receptor 1	*Slc8a2*	Solute Carrier Family 8 Member A2
Faah	Fatty Acid Amide Hydrolase	*Sod2*	Superoxide Dismutase 2
Fasn	Fatty Acid Synthase	*Srd5a2*	Steroid 5 Alpha-Reductase 2
Foxp2	Forkhead Box P2	*Srd5a3*	Steroid 5 Alpha-Reductase 3
Gabbr1	Gamma-Aminobutyric Acid Type B Receptor Subunit 1	*Tgfbeta1*	Transforming Growth Factor Beta 1
Gper1	G Protein-Coupled Estrogen Receptor 1	*Tial1*	TIA1 Cytotoxic Granule Associated RNA Binding Protein Like 1
Grin2b	Glutamate Ionotropic Receptor NMDA Type Subunit 2B	*Tnf*	Tumor Necrosis Factor
Hdac1	Histone Deacetylase 1	*Trim63*	Tripartite Motif Containing 63
Htr2a	5-Hydroxytryptamine Receptor 2A	*Zfp423*	Zinc Finger Protein 423
Ido2	Indoleamine 2,3-Dioxygenase 2		

1   INTRODUCTION

1.1   Polycystic ovary syndrome

1.1.1   Definition

Polycystic ovary syndrome (PCOS) is the most common endocrine disorder among women of reproductive age, which is characterized by clinical or biochemical hyperandrogenism (hirsutism, acne/excess of circulating androgens, respectively), oligoovulation or anovulation, and polycystic ovarian morphology (1, 2). PCOS manifests with a heterogeneous phenotype, it is the main cause of anovulatory infertility, and it is linked to metabolic, cardiovascular and mental complications in the affected women (3).

PCOS was first described by Stein and Leventhal in 1935. The investigators characterized a group of seven women that shared symptoms of menstrual irregularities, hirsutism, and enlarged ovaries with many small follicles. Treatment with bilateral ovarian wedge resection resulted in menstrual improvements and five of them became pregnant after the treatment. Of the two remaining subjects, one discontinued the follow-up and the other faced concomitant male factor infertility (4-6). Since then, many researchers have attempted to further characterize the phenotype of the syndrome, to elucidate its origins, and to apply various treatments in order to alleviate the symptoms in the affected women.

1.1.2   Diagnostic Criteria and Prevalence

Diagnostic criteria

It was more than five decades after the initial description of the syndrome, that the first formal diagnostic criteria were proposed at a conference on PCOS sponsored by the National Institutes of Health (NIH) in 1990. These criteria, which are known as the NIH criteria, require the presence of hyperandrogenism (clinical or biochemical) and ovulatory dysfunction for the diagnosis of the syndrome, excluding the presence of other disorders that lead to similar features. While the NIH criteria have been widely accepted, increasing research in the PCOS field the upcoming years, led to the refinement of the NIH criteria. In 2003, a group of experts at a conference that took place in Rotterdam, in the Netherlands, revisited the NIH criteria and decided to include features of polycystic ovarian morphology in the diagnostic criteria (2). The Rotterdam consensus is widely endorsed for the diagnosis of the syndrome and it is currently in clinical use. According to the Rotterdam criteria, at least two of the following features should be present for the diagnosis of PCOS: 1) clinical or biochemical hyperandrogenism (hirsutism/excess levels of androgens, respectively); 2) oligo-anovulation (menstrual dysfunction); and 3) polycystic ovaries (PCO) (excessive number of preantral follicles in the ovaries and/or enlarged ovaries), with the exclusion of other disorders. The use of the

Rotterdam criteria gives rise to four PCOS phenotypes, which may differ in severity, depending on the presence or not of hyperandrogenism. Of note, hyperandrogenism is evidenced to coincide with both the reproductive and metabolic abnormalities of the syndrome (7). To cope with this phenotypic heterogeneity, the Androgen Excess and PCOS (AE-PCOS) society in 2006 suggested that hyperandrogenism should be a prerequisite for the diagnosis of the syndrome, followed by either ovulatory dysfunction or polycystic ovarian morphologic features (8). The three different sets of diagnostic criteria of PCOS are summarized in Table 1, and the PCOS phenotypes that arise using the Rotterdam criteria are presented in table 2.

Table 1. Diagnostic criteria of PCOS (Adapted from (9))

Features	NIH 1990	Rotterdam 2003	AE-PCOS Society 2006
Hyperandrogenism	Hyperandrogenism required	Any two of the three features (hyperandrogenism, ovulatory dysfunction, polycystic ovarian morphologic features) required	Hyperandrogenism required
Oligo-ovulation or anovulation	Ovulatory dysfunction required	Any two of the three features (hyperandrogenism, ovulatory dysfunction, polycystic ovarian morphologic features) required	Either ovulatory dysfunction or polycystic ovarian morphologic features required
Polycystic ovarian morphologic features	Not applicable	Any two of the three features (hyperandrogenism, ovulatory dysfunction, polycystic ovarian morphologic features) required	Either ovulatory dysfunction or polycystic ovarian morphologic features required

Table 2. Four phenotypes of PCOS using the Rotterdam 2003 criteria

Phenotypes	Hyperandrogenism	Oligo/anovulation	Polycystic ovaries
Phenotype 1 ("classic" PCOS)	√	√	√
Phenotype 2 (NIH criteria)	√	√	×
Phenotype 3 (ovulatory PCOS)	√	×	√
Phenotype 4 (non-hyperandrogenic PCOS)	×	√	√

Prevalence

PCOS is a prevalent disorder that affects women of reproductive age across all races and ethnicities. Due to the different diagnostic criteria used and the high number of undiagnosed cases, the prevalence of the syndrome varies. Based on the results of a recent systematic review and meta-analysis, the prevalence reaches 6% (5-8%) worldwide using the stringent NIH criteria, while it is 10% following the Rotterdam 2003 (8-13%) and the AE-PCOS society 2006 (7-13%) criteria (10). There is no adequate literature to support geographical and racial differences in the prevalence of the syndrome (11). Yet, a small-scale study found that the prevalence of PCOS in adult normal weight Indigenous Australian women was 15% and as high as 30% when the body mass index was above 30 kg/m^2, using the NIH criteria (12). Moreover, a study in severely obese women (BMI>35 kg/m^2) found that the prevalence of PCOS was 25,6% (13).

1.1.3 Pathophysiology

The pathophysiology of PCOS is rather complex and our knowledge is incomplete partly due to the diverse phenotypic profiles of women with the syndrome.

The most common defect in PCOS is ovarian hyperandrogenism, which is present in more than 85% of women with PCOS and originates from an unexplained steroidogenic hyperactivity and leads to increased ovarian theca cell production and secretion of androgens, and ovulatory dysfunction (14). Other possible components that underlie the pathogenesis of the syndrome and lead to the clinical features of PCOS are depicted in Figure 1. Almost 50% of women with PCOS display elevated levels of luteinizing hormone (LH) along with increased LH pulse amplitude (15). LH controls the expression of gonadal steroidogenic enzymes and the secretion of sex hormones. An increased pulsatile release of gonadotropin-releasing hormone from the hypothalamus may lead to increased pulse frequency of the luteinizing hormone by the pituitary gland at the expense of follicle-stimulating hormone. This neuroendocrine defect leads to increased ovarian theca cell production and secretion of androgens and ovulatory dysfunction (16). Moreover, insulin resistance and hyperinsulinemia are present in about 30% of women with PCOS (17). The origins of insulin resistance in PCOS are of intrinsic character in most cases, while it can be a consequence of obesity in some cases. Insulin stimulates androgen production by directly stimulating the theca cells, and hyperinsulinemia in PCOS further contributes to increase of ovarian androgen production (18-20). In addition, hyperinsulinemia inhibits the hepatic synthesis and secretion of the sex hormone transporter, sex hormone binding globulin (21), which further contributes to hyperandrogenemia (22). Sympathetic nerve activity is elevated in women with PCOS and the common features of the syndrome, such as hyperandrogenism, insulin resistance and obesity contribute to increased sympathetic nerve activity in

these women (23, 24). The role of sympathetic nerve activity in the pathophysiology of PCOS will be discussed in more detail in the part II of this thesis. Finally, the interplay among the for mentioned mechanisms constitutes a vicious circle and leads to long-term health complications in the affected women (Figure 1).

Figure 1. Pathophysiology of PCOS.
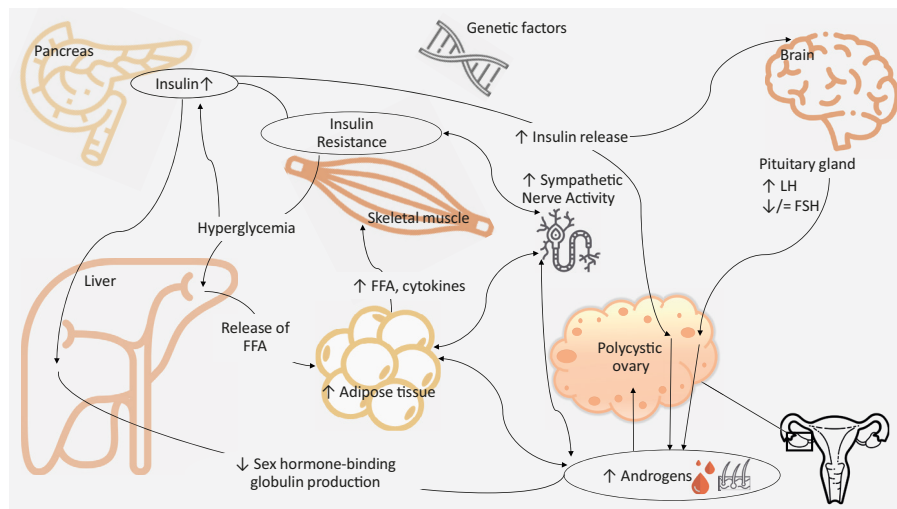
LH, luteinizing hormone; FSH, follicle-stimulating hormone; FFA, free fatty acids (The icons used in this figure were made by https://www.flaticon.com/authors/freepik)

1.1.4   Etiology

A large piece of evidence indicates that genetic, intrauterine and postnatal environmental factors are implicated in the etiology of the disorder (25). Starting with the genetic factors, familial and twin studies have revealed that the heritability of PCOS reaches the 70% and follows an autosomal dominant fashion (26-29). Despite the high heritable character of the syndrome, studies aiming to discover target candidate genes that cause PCOS have been hampered by the phenotypic heterogeneity of the syndrome itself and the complex variety of identified genes. Moreover, genome-wide association studies (GWAS) have attempted to identify loci that could be implicated in the etiology of the syndrome, but the PCOS loci that have been identified so far account for less than 10% of the heritability (30). Some candidate genes that have been associated with PCOS in Chinese and European populations by GWAS are the following: *LHCGR, FSHR, THADA, DENND1A, FSHB, RAB5B/SUOX, HMGA2, TOX3* and *INSR*. These genes are involved in hormonal regulation, insulin signaling and organ growth (29).

The intrauterine environment is an important environmental factor that has been implicated in PCOS etiology. Pregnant women with PCOS have elevated levels of androgens during the second and third trimester, which could pose the developing fetus at risk for exposure to excess androgens (31-33). Studies in animal models of PCOS, including non-human primates, sheep and rodents have shed light into the effects of *in utero* virilization in the developing embryo, and accumulating data indicate that gestational androgen excess can lead to PCOS traits in the adult offspring (34). Obesity is another crucial environmental factor that is tightly linked to PCOS. It is highly debated whether obesity per se can lead to PCOS, yet it is believed to lead to symptom manifestation in genetically susceptible individuals (14).

All in all, there is need for a better understanding of the genetic, epigenetic and environmental factors as well as the interaction among them, in order to better understand the etiology of PCOS.

1.2 Animal models of PCOS

Over the last 50 years, animal models of experimentally induced PCOS have been developed to examine the etiology of PCOS and facilitate the understanding of the complex aspects of the syndrome (34, 35). Despite the lack of an established animal model that mirrors all symptoms of the syndrome, various animal models exposed to different interventions develop a PCOS-like phenotype resembling reproductive, metabolic and behavioral traits of the syndrome. Starting with the *in utero* programming of the syndrome, prenatally androgenized non-human primates, sheep and rodents during early or late gestation have given substantial evidence that PCOS may originate from an adverse intrauterine environment with androgen excess. Prenatally androgenized rhesus monkeys and sheep are the best models to study PCOS as they closely mimic the human traits of the syndrome, including hyperandrogenism, oligo- or anovulation, and polyfollicular ovaries. Moreover, they develop many neuroendocrine, metabolic and behavioral abnormalities that are present in women with PCOS (36, 37). However, the use of these animals is expensive and requires a long experimental time due to their long reproductive cycle. Therefore, many researchers have focused their research on prenatal androgenized rodent models, since they have a short estrous cycle of 4-5 days and a short reproductive cycle. These studies have provided us with important insight into the possible mechanisms of action, yet the androgenized rodent offspring resemble partially the human PCOS phenotype (38-48). Although they display irregular cyclicity, signs of metabolic, neuroendocrine and behavioral complications, the results concerning the hyperandrogenism and the ovarian morphology are conflicting.

Moreover, animal models of neonatal and prepubertal androgen exposure have been generated to study the role of hyperandrogenemia in the development of PCOS-like features (49-56). Studies in monkeys exposed to slightly elevated testosterone from prepuberty showed modest neuroendocrine defects, with no major changes in metabolic and ovarian features (50, 51). On the other hand, prepubertal exposure of mice to androgens have shown robust ovarian and metabolic changes (53).

Interventions including estrogen, letrozole, antiprogesterone agent administration during critical developmental stages as well as constant light exposure, D-galactose and valproic acid are some additional examples of studies in rodents that tried to resemble the human PCOS (57-61). Finally, genetic mouse models have been developed to assess the role of specific genes in the pathophysiology of the syndrome. These models include the 17NF transgenic mice that overexpress nerve growth factor selectively in the theca-interstitial cells (62), and will be extensively discussed in the part II of this thesis book, mice with targeted overexpression of luteinizing hormone (63), human chorionic gonadotropin (64), plasminogen activator inhibitor-1 (65), prohibitin (66), and mice lacking leptin and insulin receptors in pro-opiomelanocortin neurons (67).

The animal models have tremendously helped us to study and understand some aspects of the pathophysiology of the syndrome; yet, it is questionable to which extent we can translate these findings into the human condition. Taking into consideration the lack of an ideal animal model to study PCOS, it is crucial for the researchers in the field to choose the most appropriate model for the study of specific aspects of PCOS.

1.3   This thesis

The overall scope of the current thesis is to provide novel insight into the developmental origins of PCOS, focusing on the impact of an adverse intrauterine environment on PCOS-related outcomes in the offspring.

The thesis consists of two parts, Part 1 and Part 2. Part 1 describes the studies I and II and examines how adverse environmental factors, including maternal obesity and maternal androgen excess, impact on behavioral health in the offspring and whether changes in behavior can be transmitted transgenerationally, using the prenatally androgenized mouse model. Moreover, it examines if PCOS in mothers increases the risk of anxiety disorders in the daughters and sons. Part 2 describes studies III and IV and examines 1) the impact of maternal androgen excess on cardio-metabolic outcomes in the offspring, along with the effects of postnatal androgen excess on the cardiovascular system (study III), and 2) the impact of ovarian sympathetic hyperactivity on the development of PCOS-like features, including developmental, reproductive and metabolic complications (study IV). Studies III and IV employ three different mouse models of PCOS to answer these questions.

2 PART 1: PCOS AND MENTAL HEALTH DISORDERS

2.1 Background

Mental health disorders comprise a wide range of mental complications, which manifest with different symptoms. General features are abnormal thoughts, emotions and behavior. Mental health disorders are among the leading causes of ill-health, affecting about 25% of the population worldwide (68). Anxiety disorders are the most common mental health disorders, they are characterized by excessive fear or anxiety, and affect women more than men (69, 70).

Women with PCOS are at increased risk of developing mental disorders (71-76). A recent review showed that 36.6% of women with PCOS have anxiety symptoms compared to 8.5% of women without the syndrome (77). Another systematic review and meta-analysis found that the odds ratio of having moderate/severe anxiety or depression are 6.30 and 3.25 in women with PCOS, respectively, after adjustment for BMI (73). Despite the well-documented evidence indicating that women with PCOS have a greater prevalence of anxiety symptoms, little is known about the underlying mechanisms and risk factors that could trigger these symptoms. A limited number of studies have investigated the correlation between the clinical/biochemical features of PCOS and anxiety symptoms and the outcomes are inconsistent. To start with, circulating testosterone and hirsutism, which are observed in the vast majority of women with the syndrome, have shown no or a weak positive correlation with anxiety symptoms in small-scale studies (73, 78, 79). Moreover, overweight and obesity, which are common features of PCOS, have been correlated with depression, but the correlation between BMI and anxiety is unclear. Although many studies showed an association between PCOS and anxiety disorders, independently of BMI (79), there are a few studies showing correlation between anxiety disorders and BMI in this population (80, 81). Last but not least, insulin resistance has not been associated to anxiety in women with PCOS (77). Overall, the most common features of PCOS do not seem to account for the increased rates of anxiety disorders in the affected women.

An adverse intrauterine environment with androgen excess may lead to abnormal fetal development and has been evidenced to affect the brain function and behavior in the offspring later in life. In particular, increased fetal testosterone, measured in the amniotic fluid, was found to induce changes in the reward system of the assessed boys at the age of 10 (82). Moreover, children of women with PCOS, which are potentially exposed to higher levels of androgens during pregnancy, are at increased risk to develop autism spectrum disease and attention deficit hyperactivity disorder, with girls being more susceptible than boys (71, 83, 84). Moreover, in paper II of the current thesis, we showed that daughters of women with PCOS are also at increased risk for anxiety disorders. To explore whether these behavioral changes in the children of women with PCOS are due to environ-

mental factors and/or genetic factors, Cesta *et al*. compared PCOS-exposed off-spring to unrelated non-PCOS-exposed offspring and non-PCOS-exposed cousins (85). They found that PCOS-exposed offspring, especially girls, were at greater risk to develop attention-deficit/hyperactivity disorder, autism spectrum disorders, Tourette's disorder and chronic tic disorders, compared to both unrelated offspring and cousins that were not exposed to maternal PCOS. These findings strengthen the hypothesis that maternal androgen excess can lead to behavioral changes in the offspring later in life, with or without genetic influences. Interestingly, the studies mentioned above show a stronger impact of maternal androgen excess on behavioral changes in girls rather the boys. The factors that drive this sexually dimorphic response are not fully understood, but it is hypothesized that *in utero* androgen excess "masculinizes" the developing female brain by permanently reorganizing brain neural systems and leading to "hyper-masculine" behavioral traits that are found more often in boys than girls (e.g. attention-deficit/hyperactivity disorder and autism spectrum disorders) (86, 87).

Studies in animal models have investigated how androgens affect brain function and behavior. In addition, the prenatally androgenized models of PCOS have been used to investigate the effects of *in utero* androgen excess in offspring brain function and behavior and tried to elucidate the involved mechanisms. Starting with the direct effects of chronic androgen exposure in female mice, it is shown that they develop anxiety-like behavior, an effect that is associated with increased mRNA expression of corticotropin releasing factor in the central nucleus of the amygdala (88). In addition, in a rat model of PCOS, continuous administration of dihydro-testosterone led to anxiety-like behavior, which was accompanied by decreased basal morning and evening plasma corticosterone levels (89). With regard to the impact of *in utero* exposure to androgens, we and others have demonstrated that maternal androgen excess leads to anxiety-like behavior in the offspring, especially the female offspring (46, 47, 90, 91). Prenatal testosterone androgenization in female rats led to downregulation of androgen receptor, and upregulation of serotonergic and GABAergic genes in the amygdala (47), while another study found an increased hippocampal protein expression of brain-derived neurotrophic factor and decreased number of neuropeptide Y and parvalbumin immunoreactive cells (90). Using the prenatal dihydrotestosterone androgenized mouse model (study I in the thesis book), combined with maternal obesity and/or offspring obesity, we showed that female offspring exposed to maternal androgens had dysregulated gene expression of the adrenergic receptor 1 beta and the corticotrophin releasing hormone 2 in the amygdala, and dysregulated gene expression of the corticotropin-releasing hormone and its receptors in the hypothalamus. A more detailed description and discussion of the results for paper I will follow in sections 2.4 and 2.5.

Overall, clinical and pre-clinical studies strongly indicate that mental health disorders in women with PCOS may originate during embryonic development due to androgen excess. Further research is needed to reveal the molecular mechanisms that are involved in this "programming".

2.2   Aims (Studies I and II)

The aims of the first two studies in the thesis were:

Study I: To investigate the distinct or combined effects of 1) *in utero* exposure to androgens, 2) maternal obesity, and 3) offspring obesity, on anxiety-like behavior in adult female and male mouse offspring; and to identify candidate genes that are implicated in anxiety-like behavior.

Study II: To investigate whether maternal PCOS increases the risk of anxiety disorders in daughters and sons, using a Swedish national register-based cohort study; to investigate whether maternal *in utero* exposure to androgens and/or maternal obesity leads to transgenerational transmission of anxiety-like behavior in adult female and male mice and to identify candidate genes that are involved in these behavioral changes.

2.3   Materials and Methods in studies I and II

2.3.1   Ethical considerations

All animal experiments in paper I and II were approved by the Stockholm Ethical Committee for Animal Research (Paper I: 121-16, Paper II: 10798-2017) in accordance with the legal requirements of the European Community and the directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes.

The register-based study in Paper II complies with the ethical standards of the relevant national and institutional committees on human experimentation and with the Helsinki Declaration of 1975, as revised in 2008. This study was approved by the regional ethical review board in Stockholm, Sweden (diary number 2013/862-31/5; 2016/1214-32). The requirement for informed consent was waived because the study was register-based, and the included individuals were not identifiable at any time.

2.3.2   Swedish national register-based cohort study

The results in this study were generated from our collaborators Carolyn Cesta (Department of Medicine, Centre for Pharmacoepidemiology, Karolinska Institutet) and Mina Rosenqvist (Department of Medical Epidemiology and Biostatistics, Karolinska Institutet). A summary of the data sources and the analysis methods are presented in Table 3.

Table 3. Summary of the data sources and the analysis methods included in Paper II.

| Population	Data sources – Swedish Population Registers	Outcome	Study Design and analysis method
All children born in Sweden from 1995 to 2007 to mothers diagnosed with PCOS (n=12,955) and up to 10 matched mothers without PCOS from the general population.	• Swedish Medical Birth Register • National Patient Register • Prescribed Drug Register • Total Population Register • Migration Register • Cause of Death Register	Diagnoses of anxiety recorded in the children after age of 6.	Matched Cohort Study Stratified Cox regression models

2.3.3 Animal models and study designs

C57BL/6J mice were obtained from Janvier labs, were housed under a 12 h light–dark cycle, with controlled conditions and had free access to water and food.

Paper I:

In order to generate prenatally androgenized mice combined with maternal obesity, 12-week-old female mice were divided into two diet groups. The composition of the high-fat/high-sucrose (HFHS) diet was 17 kcal% protein, 43 kcal% carbohydrate, and 40 kcal% fat, and together with the food pellets, mice had also free access to a 20% sucrose solution for drinking, supplemented with a vitamin mix of 10 g/4000 kcal and a mineral mix of 35 g/4000 kcal. We chose to give this HFHS diet, in order to mimic the western diet that is characterized by high intake of fat and sugar. The composition of the control diet was 17 kcal% protein, 73 kcal% carbohydrate, and 10 kcal% fat.

To generate prenatally androgenized mice (PNA), we followed an established protocol that is widely used by researchers in the field (38, 39, 92). Using this model, we mimic the PCOS pregnancies with increased circulating androgen levels during the last trimester of pregnancies (31, 32). After 10 weeks on the diet, a female mouse on proestrus or estrus phase was placed with a male mouse in the afternoon and a plug the morning after mating was considered gestational day (GD) 0.5. On GD 16.5 pregnant dams were injected in the interscapular area with 100 µl vehicle or 250 µg DHT dissolved in a mixture of 5 µl benzyl benzoate and 95 µl sesame oil.

To investigate how three environmental factors (maternal obesity, maternal androgen excess and offspring obesity) interact with each other and affect anxiety-like behavior in the female and male offspring, we generated 16 groups of offspring:
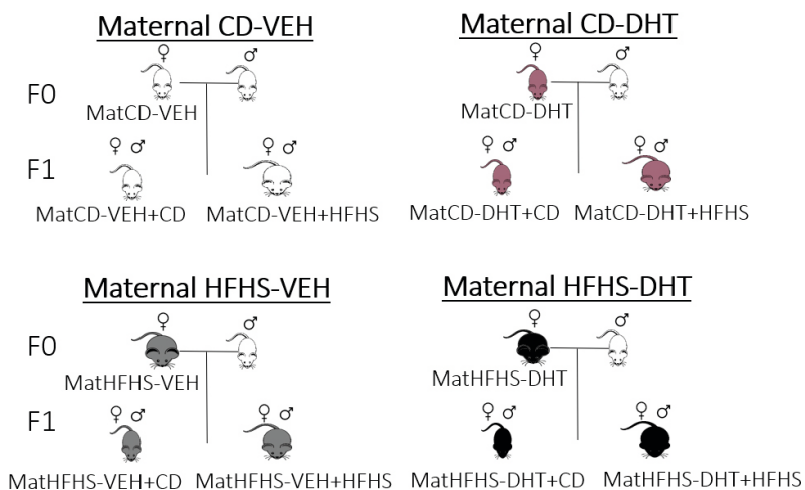
Paper II:

In Paper II we used the same PNA mouse model combined with maternal obesity, as in Paper I, with some refinements. Due to practical difficulties using the HFHS diet in Paper I, we used here a HFHS diet that combines high intake of fat and sucrose in the same food pellet, without the need of a sucrose bottle (93). The generation of PNA mice follows the same protocol as in Paper I.

To investigate the transgenerational effects of maternal obesity and maternal androgen excess on anxiety-like behavior, we created three generations of mice. To investigate transgenerational epigenetic inheritance, we need to assess a generation that is not directly exposed to the environmental stimuli (94). In the case of maternal lineage (F0), both the developing first generation (F1) fetus and the primordial germ cells of second generation (F2) are directly exposed to the F0 environmental stimuli. This transmission is referred as intergenerational epigenetic inheritance. The transgenerational epigenetic inheritance can be studied in the third generation that is not directly exposed. On the other hand, in the case of paternal lineage (F0), the transgenerational epigenetic inheritance can be studied in the F2 generation of mice (Figure 2).
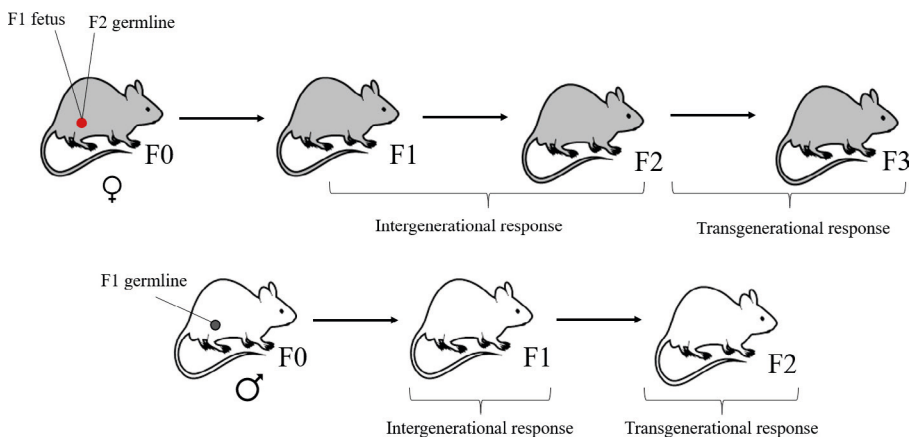
Figure 2. Illustration of intergenerational and transgenerational epigenetic inheritance of environmental stimuli in F0 maternal germline and F0 paternal germline.

In paper II, we were also interested to investigate the transmission of anxiety-like behavior in the male germline. Therefore, we mated the F1 males and generated the second and third generation of male mice.

The mating scheme and the groups that were studied are presented in Figure 3:
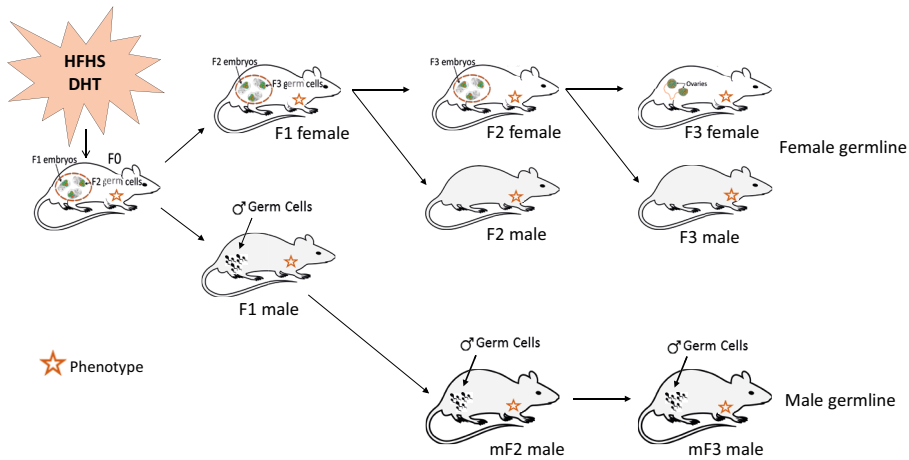
Figure 3. Schematic illustration of experimental design. *Prior to mating with male mice fed a control diet, F0 mothers were fed a control diet or a high fat-high sucrose (HFHS) diet for 6 weeks. During the embryonic days, E16.5-E18.5, pregnant mice were injected subcutaneously with dihydrotestosterone (DHT) or vehicle resulting in four lineages; CD+Vehicle (control); CD+DHT (androgenized); HFHS+Vehicle (obese); and HFHS+DHT (obese and androgenized). F1 female and F1 male offspring were mated with unrelated males and females fed CD, respectively, to generate F2 and thereafter to generate F3 offspring. Male germline refers to mF2 and mF3 male offspring from F1 males. F2 and F3 males are siblings to F2 and F3 female offspring (female germline).*

2.3.4   Estrous cycle and anogenital distance

Mice are mammals and their female genital system is highly dynamic and influenced by hormones, similarly to human females. The histological appearance and function of the genital system is similar between the two species. Mice have an estrous cycle of 4-5 days, which is divided into 4 stages (proestrus, estrus, metestrus, diestrus), and corresponds to the human menstrual cycle. Disruption of estrous cycle in mice could reflect reproductive dysfunction. Here we used the vaginal cytology method to identify whether the mice have a normal estrous cycle or if they are acyclic. Three types of cells (nucleated epithelial cells, cornified epithelial cells, and leukocytes) are identified in the estrous cycle, and the relative proportion of each cell type determines each estrous phase. The proestrus phase is characterized by the presence of nucleated and some cornified epithelial cells, followed by the estrus phase with predominantly cornified epithelial cells. The cycle continues with the metestrus phase, which consists of a mixture of three cell types and ends with the diestrus phase, where leukocytes is the predominant cell type (95).

Women with PCOS and their daughters have longer anogenital distance (AGD) (96-98). AGD is evidenced to be a reliable newborn biomarker of *in utero* androgen exposure. Therefore, in our studies, we measured the AGD in 3-week-old pups in order to confirm fetal androgen exposure.

2.3.5 Assessment of anxiety-related behavior in mice

To assess anxiety-like behavior in the mice, we used two behavioral tests, the elevated plus maze and the open field.

Elevated plus maze is a well-established behavioral assay that has been widely used for assessment of anxiety-like behaviors in rodents (99). The maze consists of four arms that form a plus shape, of which two are enclosed and two are open. This test assesses anxiety responses in rodents based on their preferences for dark, enclosed spaces and their fear of heights and open spaces. Rodents are placed at the center of the maze and their activity is recorded. Rodents that spend most of the time in the enclosed arms and avoid the open arms display anxiety.

Open field is also a widely used behavioral test to assess anxiety in rodents (100). In most cases it is a square box with an empty large area that creates the feeling of openness. The test relies on the aversion of rodents for open and unknown environments. The box is divided into two parts, the center and periphery. Rodents are placed at the center of the open field and their activity is recorded. Rodents that tend to spend more time closer to the walls of the box and avoid exploring the center display higher anxiety.

2.3.6 Gene expression analysis in the amygdala and hypothalamus

For papers I and II, we decided to follow a targeted approach, where we selected to assess several genes that have been previously implicated in anxiety. This approach can provide us with important information, yet it does not provide a comprehensive picture of whole genome expression. Real-time reverse transcription-polymerase chain reaction (RT-PCR) using the TaqMan Gene Expression Assays was used to measure gene expression in the amygdala. RT-PCR was also used to assess the gene expression of corticotropin-releasing hormone and its receptors in the hypothalamus.

2.3.7 Enzyme-linked immunosorbent assay (ELISA)

ELISA was used to measure the serum levels of testosterone and corticosterone in Paper I. This method detects and quantifies proteins in testing samples, by using antibodies that detect the protein of interest immobilized on the surface of a well.

2.3.8 Statistical analysis

In the register-based cohort study, associations between maternal PCOS and offspring anxiety were estimated as hazard ratios with 95% confidence intervals using stratified Cox regression models with attained age as the underlying time scale. In addition to the maternal matching criteria, potential confounding variables were

adjusted for including offspring sex and year of birth, maternal age at child's birth, maternal education, maternal region of birth, and maternal and paternal lifetime history of psychiatric disorders (adjusted Model). Robust standard errors were used to account for dependence between observations since several children from the same family were included in the study population. The analysis was conducted first for all offspring combined, and then stratified by offspring sex.

The statistical analyses in the mouse experiments were performed using the SPSS software in Paper I and SPSS software and GraphPad in Paper II. One-way or two-way or three-way ANOVA were used to analyze our variables. Using two-way and three-way ANOVA, we were able to determine the main effects or the interactions of two or three independent variables, respectively. The detailed description of the statistical tests used in each experiment, can be found in the respective papers. The α level was set at $P \leq 0.05$.

2.4   Results of studies I and II

2.4.1   Paper I: Maternal androgen excess and obesity induce sexually dimorphic anxiety-like behavior in the offspring

In utero exposure to androgens led to anxiety-like behavior in adult female, but not male offspring. On the other hand, maternal obesity increased anxiety-like behavior only in male offspring. The expression analysis of target genes involved in anxiety within amygdala and hypothalamus also showed a sexually dimorphic pattern. Female offspring exposed to DHT showed an upregulation of *Adra1b* and *Crhr2* in the amygdala, and dysregulation of *Crh* and its receptors, *Crhr1* and *Crhr2*, in the hypothalamus. Male offspring exposed to maternal obesity showed an upregulation of epigenetic markers in the amygdala and dysregulation of *Crh*, *Crhr1* and *Crhr2* in the hypothalamus, among other genes. While maternal diet did not lead to anxiety-like behavior in the female offspring, it did have an impact on gene expression in the amygdala of these mice. Finally, we showed that offspring diet did not have a direct impact on anxiety-like behavior in the offspring but led to dysregulated expression of many genes within the amygdala. This finding implies that offspring diet is an important environmental factor that acts synergistically with the maternal factors (maternal obesity and maternal androgen excess) for the manifestation of the anxiety-like behavior in the offspring. A detailed description of the dysregulated genes in the amygdala and hypothalamus can be found in Tables 4-5:

Table 4. Dysregulated genes in the amygdala of female and male offspring. Main or interaction effects of maternal androgen excess and maternal and offspring diet were calculated with 3-way ANOVA.*, indicates interaction effects.

Genes	Females	Males
Neurotrophic factors and related receptors		
Bdnf	Maternal diet	Maternal*offspring diet
Ntrk2	Maternal diet	-
Ngfr	-	Maternal*offspring diet
Ntrk1	Maternal diet, Offspring diet	-
Nrg1	DHT exposure*offspring diet, Maternal diet	DHT exposure *offspring diet
Erbb4	Maternal diet	Maternal diet
Genes involved in key neuronal networks		
Gabbr1	Maternal diet	Maternal*offspring diet
Htr2a	Offspring diet	-
Slc6a4	Maternal*offspring diet	-
Nr3c1	Maternal*offspring diet	-
Crhr2	DHT exposure*offspring diet	Maternal diet
Adra1a	-	Maternal*offspring diet
Adra1b	DHT exposure*maternal diet	-
Grin2b	Maternal diet	-
Faah	-	Maternal*offspring diet
Ido2	-	Maternal*offspring diet
Androgen and Estrogen receptors		
Ar	Maternal diet	-
Gper1	Maternal diet	Maternal*offspring diet
Ers1	DHT exposure*offspring diet, Maternal diet	-
Metabolic genes		
Sirt1	DHT exposure*offspring diet, Maternal diet	Offspring diet
Insr	DHT exposure*maternal diet	Maternal*offspring diet
Irs1	-	Maternal*offspring diet
Lepr	Maternal diet	
Il1r1	Maternal diet, Offspring diet	Maternal diet
Histone mediators		
Ep300	-	Maternal diet, Offspring diet
Hdac1	-	Maternal*offspring diet
Crebbp	-	Maternal*offspring diet

Table 5. Dysregulated genes in the hypothalamus of female and male offspring. Main or interaction effects of maternal androgen excess and maternal and offspring diet were calculated with 3-way ANOVA. *, indicates interaction effects.

Genes	Females	Males
Crh	DHT exposure, Offspring diet	Maternal*offspring diet
Crhr1	DHT exposure *maternal diet	DHT exposure *maternal diet
Crhr2	DHT exposure	DHT exposure *maternal diet

2.4.2 Paper II: Prenatal androgen exposure causes a sexually dimorphic transgenerational increase in offspring susceptibility to anxiety disorders

Here we showed using a Swedish register-based cohort study that daughters of women with PCOS, but not sons, have a 78% increased risk of being diagnosed with anxiety compared to daughters of women without PCOS. We further explored whether maternal androgen excess and/or maternal obesity leads to anxiety-like behavior in male and female mice offspring and whether these two prominent environmental stimuli in the mother can lead to transgenerational transmission of anxiety-like behavior. We found, similarly to the human data, that only female offspring (F1) but not male offspring (F1), displayed an anxiety-like behavior due to maternal androgen excess with or without obesity in the first generation. Moreover, we showed that this anxiety-like behavior was transmitted into the third generation of female mice (F3), although no anxiety-like behavior was observed in the second generation (F2). No changes in anxiety-like behavior were observed in all three generations of male offspring following the female germline (F1-F3). Further, we investigated whether the male germline, here the first-generation male offspring (F1), transmit anxiety-like in the subsequent male generations. We found that only the third generation of male offspring (mF3) generated from the androgenized lineage and the obese lineage showed signs of anxiety-like behavior. These transgenerational behavioral changes were associated with gene expression changes within the amygdala. In the third generation of female offspring (F3), five genes (*Camk2n1*, *Cacna2d1*, *Slc17a7*, *Btg2*, and *Dbh*) were downregulated in the androgenized and in the obese lineages and three genes (*Foxp2*, *Tial1*, *Adora2a*) were upregulated only in the obese lineage. Lastly, the third generation of male offspring from the obese male lineage (mF3) had downregulated expression of the *Btg2*, *Comt* and *Otx4*.

2.5 Conclusions

In Papers I and II, we observed a prominent sexually dimorphic behavioral response to an adverse intrauterine environment with androgen excess and obesity. While it is not feasible to study transgenerational transmission in humans – at least today – we observed in a mouse model of PCOS combined with maternal obesity that these adverse conditions can lead to behavioral changes in the first generation of female offspring and in the third generation of female offspring that was not directly exposed. These findings suggest a transgenerational epigenetic inheritance, which could be mediated through permanent changes in epigenetic mechanisms of somatic and germ cells. Another intriguing finding is the different behavioral response in males and females. Why do female offspring are more affected by maternal androgen excess than male offspring? It is believed that male fetuses can adjust testicular androgen production and prevent high levels of androgens prenatally. This hypothesis is further supported by the finding that maternal testosterone levels correlate with the testosterone levels of daughters but not sons (101). Further research is needed to increase our understanding of the mechanisms that lead to such sexual differences.

Obesity is a common feature of PCOS, and we showed in the mouse studies that maternal obesity affects anxiety-like behavior in males more than in females. However, the gene expression analysis in the amygdala revealed changes due to maternal obesity in both sexes. Therefore, obesity is an important environmental factor that may reorganize brain neural systems in the offspring and lead to behavioral changes later in life.

In these studies, we followed a targeted approach to identify the involved genes that may mediate these behavioral changes. Although we did identify a dysregulation of numerous genes, there is need for a whole genome approach that will provide a deeper understanding of the involved pathways. Moreover, separate analysis of the amygdala nuclei could provide a more precise description of the involved mechanisms.

Finally, these findings highlight the need for screening the children of women with PCOS for mental health disorders and underscore the detrimental effects of maternal obesity in offspring health.

3 PART 2: PCOS AND CARDIO-METABOLIC DISTURBANCES

3.1 Background

3.1.1 Cardiovascular profile in PCOS

Cardiovascular diseases are the most common cause of death worldwide and they are related to dysfunction of the heart and blood vessels. A number of risk factors, such as obesity, hypertension, type 2 diabetes mellitus (T2DM) and abnormal lipid levels may increase the risk for cardiovascular diseases (102).

Studies in women with PCOS have demonstrated that they are at an increased risk for cardiovascular diseases (103-107). Whether this increased risk is associated to the syndrome itself, or to the metabolic complications that are often present in women with the syndrome is unclear. Assessment of echocardiographic parameters have indicated structural alterations in young women with PCOS, including increased left ventricular mass and left atrial diameter (108, 109). Also, women with the syndrome are more prone to develop endothelial dysfunction, especially those who comorbid with insulin resistance and hyperandrogenemia (110, 111). Another study showed an increased intima-media thickness of common carotid and femoral arteries in women with the syndrome, which remained significant after adjustment for known risk factors (104, 112). In addition, women with PCOS were shown to have increased prevalence of coronary and aortic calcification, a finding that was associated with insulin resistance (113). On the other hand, another study did not show significant differences in coronary artery calcium or abdominal aortic plaque in women with PCOS (114). Increased levels of fibrinogen and plasminogen activator inhibitor 1 were also found in women with PCOS after adjustment for confounding factors, suggesting a prothrombotic state (115). Finally, hypertension is evidenced to be more prevalent among women with PCOS, a finding that was independent of age (116-119).

Several studies have also investigated the impact of maternal PCOS on offspring cardiometabolic health. A recent study compared the cardiovascular and metabolic profile of young children of women with PCOS (2 groups: 3-4y and 7-8y) to a reference population (120). Their findings revealed that offspring from women with PCOS have a distinct cardiac and metabolic profile. Among the differences, they showed that the younger group had increased pulse pressure and left ventricle internal diameter, yet the latter was within the normal range. The older group of children from women with PCOS had higher carotid intima-media thickness compared to the reference group (120). In addition, another study showed that young adult daughters of women with PCOS, who had themselves a PCO morphology, displayed increased diastolic and mean arterial pressure (121). The clinical importance of these findings is not known and need further investigation. Yet, these

findings suggest that maternal PCOS may predispose the offspring to cardiometabolic complications, and the possible mechanisms of action await investigation.

Animal studies have tried to shed light into the link between PCOS and cardiovascular abnormalities. We and others have investigated the impact of androgens on cardiovascular system and how a hyperandrogenic maternal environment affect the cardiometabolic health of the offspring. A study investigating the impact of perinatal exposure to DHT in the offspring, showed vascular dysfunction in old mice (20-month-old) and this was associated with estrogen receptor β suppression in endothelial progenitor cells early in life (122). Moreover, prenatally androgenized female rats exposed to testosterone *in utero* exhibited increased blood pressure in adulthood, and the authors suggested that androgens control the expression of protein kinase C δ (PKCδ) in the mesenteric arteries by increasing PKCδ-mediated vasoconstriction and hypertension (123). Another study in a prenatally androgenized sheep model also found gene expression changes in the cardiac tissue and increased cardiomyocyte size after histological investigation (124). Our study (Paper III) further explored the mechanisms that contribute to the development of cardiovascular dysfunction in PCOS and the results will be discussed in detail in the section 3.4.1.

3.1.2 Metabolic profile in PCOS

Women with PCOS are more prone to become overweight or obese (25, 125). The prevalence of obese women with PCOS ranges between 50-80%, it is analogous to the prevalence of obesity in each country, and aggravates many features of PCOS, including hormonal, reproductive and metabolic parameters (3). A highly debated question is whether obesity per se could promote the development of PCOS. A recent study using a diet-induced obese rat model, showed that animals exposed to a chronic high-fat high-sucrose (HFHS) diet developed signs of PCOS, including polycystic ovaries and irregular estrous cycles (126). Moreover, studies in obese women with PCOS have shown that substantial weight loss improves some aspects of the syndrome (125, 127). Thus, while there is a clear link between PCOS and obesity, the exact interplay between them needs further investigation.

Several studies have reported impaired adipose tissue function in PCOS. To start with, enlarged adipocytes in the subcutaneous adipose tissue of women with PCOS have been found when compared with age and BMI paired matched controls (128). This adipocyte enlargement could indicate a dysfunctional lipolytic activity, which can promote fat accumulation in peripheral tissues and subsequently lead to insulin resistance. A study in young non-obese women, showed a stark increase in the catecholamine-stimulated lipolysis of visceral fat cells of women with PCOS compared to controls, with no differences in adipocyte size. This increased lipolytic rate was attributed to increased function of the protein kinase A-hormone

sensitive lipase complex (129). Moreover, larger adipocytes are associated with altered adipokine secretion, and women with PCOS have lower levels of circulating adiponectin and increased levels of proinflammatory cytokines (128, 130). These findings have led to the hypothesis that a low-grade inflammation may be present in PCOS (131).

Insulin resistance is a central feature of PCOS, and studies have shown that 30-40% of women with the syndrome present with glucose intolerance and 10% of them develop T2DM by the 4th decade of their life (132-134). Moreover, another study showed that PCOS can lead to an earlier diagnosis of T2DM by 4 years (135). Obesity, especially visceral adiposity, is the best predictor of insulin resistance in PCOS and a recent review showed that increased BMI can augment a reduction of insulin sensitivity by 15% in PCOS compared to non-PCOS subjects (17, 136, 137). Further, enlarged adipocytes together with low circulating adiponectin levels are two strong markers for insulin resistance in women with PCOS (128).

3.1.3 Sympathetic activity and PCOS

The most common features of PCOS, including hyperandrogenemia, hyperinsulinemia, obesity and cardiovascular diseases, have all been associated with an increased sympathetic nerve activity (138). Women with PCOS have enhanced sympathetic nerve activity, as indicated by direct muscle sympathetic nerve activity recordings and decreased vagal activity (23, 24, 139, 140). In addition, other studies in women with the syndrome, revealed an increased catecholaminergic density of nerve fibers in the ovaries (141), increased levels of dopamine and norepinephrine in the follicular fluid and alterations in noradrenaline deamination (142, 143). These findings indicate changes in the sympathetic tone of these women. Animal studies have shown that an increase of ovarian nerve growth factor levels precedes the development of PCOS-like features (144, 145). Another study that investigated the role of peripheral noradrenergic neurons in the development of the syndrome, found that elimination of these neurons by pharmacological procedures prevents ovulatory dysfunction and hyperandrogenism in a mouse model of PCOS. Moreover, in the same study they demonstrated that sympathetic denervation can restore ovulation, but not hyperandrogenism (146). Other studies have showed an altered catecholamine content in the ovaries of a PCOS-like mouse model, with increased levels of noradrenaline and down-regulation of β-adrenoreceptors (147-149). Overall, these data suggest that sympathetic hyperactivity may play an important role in the ovarian dysfunction in PCOS and provide support for a neural involvement in the pathophysiology of PCOS

3.1.4   Nerve growth factor (NGF) and PCOS

NGF is a neurotrophic factor that was discovered in the early fifties and since then numerous studies have revealed its involvement in a broad spectrum of biological activities (150). NGF can act in both neuronal and nonneuronal cells and it is evidenced to be an important mediator of crosstalk between systems that control homeostatic functions (151). To name a few functions, NGF is critical for the development and maintenance of the peripheral sympathetic and some sensory neurons, it is involved in immune cell function, endocrine function and behavioral responses. Within the ovary, it plays a role in follicle maturation and ovulation (152), as indicated by studies in the ovaries of rodent (153, 154), goat (155, 156) and sheep models (157). In addition, it contributes to the production of steroids in the ovary, as observed in rodents (158), cows (159) and humans (160).

A lack or excess of NGF in target sites may initiate perturbations. Looking into the adverse effects of NGF imbalance within the ovary, rodent studies have shown that lack of NGF impairs the early follicular development (161) and causes infertility (162). An excess of ovarian NGF in transgenic mice (17NF mice) causes increased ovarian sympathetic tone, irregular cyclicity, impaired fertility, enhanced ovarian sex steroid production and increased granulosa cell apoptosis (62, 163). Interestingly, it has been shown that women with PCOS have increased levels of NGF in the follicular fluid and their granulosa cells produce more NGF compared to women without the syndrome (62, 164).

These findings on the role of NGF in ovarian function, led us to further investigate the 17NF transgenic mouse model with overproduction of ovarian NGF, with goal to elucidate the possible crosstalk between ovarian disruption and the other complications that are present in women with PCOS. These data are part of Paper IV and will be described in detail in the following sections.

3.2   Aims (Studies III and IV)

The aims of the study III and IV in the thesis were:

Study III: To investigate the effects of maternal androgen excess with or without maternal obesity on the metabolic and cardiac profile of female offspring. Also, to investigate the impact of continuous dihydrotestosterone exposure on the cardio-vascular system using a mouse model of PCOS.

Study IV: To investigate the effects of ovarian nerve growth factor excess on embryonic development, as well as on reproductive and metabolic function in adult life, using a transgenic mouse model that resembles PCOS-like features.

3.3 Materials and Methods in papers III and IV

3.3.1 Ethical considerations in papers III and IV

All animal experiments in paper III and IV were approved by the Stockholm Ethical Committee for Animal Research (Paper III: 10798-2017, Paper IV: 865-2019) in accordance with the legal requirements of the European Community and the directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes.

3.3.2 Animal models and study design in paper III

In Paper III, we used two mouse models of PCOS. The first one is the PNA mouse model refined with maternal obesity and was generated exactly as the PNA mouse model in paper I. In paper III we followed only the female offspring that were fed with a control diet. Moreover, we studied in a separate batch of PNA mice, the effects of maternal androgen exposure on the hearts of newly born female pups (postnatal day 1). Finally, to study the effects of dihydrotestosterone on the cardiovascular profile in a PCOS context, we used a mouse model of PCOS that is generated by continuous exposure to dihydrotestosterone from adolescence. This is an established mouse model of PCOS and is known to develop reproductive and metabolic complications within 90 days of dihydrotestosterone exposure (53). In brief, 4-week-old female mice received an implant containing dihydrotestosterone, or blank, or dihydrotestosterone+flutamide (androgen receptor blocker). In our study, mice were exposed for 7 weeks, as we wanted to assess cardiac function, without the possible confounding effects of disturbed glucose metabolism on cardiac function. Flutamide is a synthetic nonsteroidal drug that binds to the androgen receptor and prevents androgens, including dihydrotestosterone, to bind to the same receptors. In women with PCOS, flutamide is used to treat hirsutism (165). Here, it was used to investigate whether it can prevent the possible adverse effects of dihydrotestosterone on the cardiovascular system.

3.3.3 Methods used in Paper III

3.3.3.1 Reproductive and Metabolic assessment

The reproductive function was assessed with the estrous cyclicity assessment as described in the first part (Study I). We assessed body composition using dual-emission X ray absorptiometry (DEXA) in the PNA mice and echoMRI in the DHT-implanted mice. Both tests provide accurate data regarding the lean mass and fat mass of the testing animals. DEXA method requires the anesthesia of the testing animal, which is not optimal, while echoMRI is quicker and can be performed in non-anesthetized animals. Therefore, once we received access to an echoMRI, we chose this method for testing. Moreover, we assessed glucose metabolism in the testing mice with an oral glucose tolerance test and an insulin tolerance test.

3.3.3.2 Cardiovascular assessment

Blood pressure was assessed in the PNA mice by using the Coda High Throughput Non-invasive Tail Monitoring System. The tail cuff method is non-invasive, and it is widely used for blood pressure measurement is rodents. However, it is not the gold standard for continuous blood pressure and heart rate recordings in awake animals, as it lacks high sensitivity. Radio telemetry is a more sensitive method for blood pressure measurement, but it requires surgery and implantation of transmitters in the abdomen or subcutaneously, which could potentially influence the results in our model of PCOS.

Mice were subjected to transthoracic echocardiography for cardiac function assessment. This test provides information about functional and structural changes in the heart and in our studies, it was performed by expertized personnel. We also measured the cardiomyocyte size by histological examination. Histology provides additional information about the structure of the tissue and identifies possible changes at the level of the cell – here the cardiomyocyte.

Finally, we assessed vascular function using wire myography. Myography is a technique used to assess functional responses and vascular reactivity of small resistance arteries. Here, mesenteries arteries were mounted in a myograph and after normalization, their contractile and vasodilator properties were assessed.

3.3.3.3 NADPH oxidase activity in the left ventricle of the heart

NADPH oxidase, a transmembrane enzyme complex, drives the production of reactive oxygen species. Therefore, the activity of NADPH oxidase is critical for the production and availability of reactive oxygen species in our body. A dysregulation of NADPH oxidase activity is evidenced to play an important role in the onset of cardiovascular complications (166), so we measured its activity in the left ventricle of the heart in our PNA mice.

3.3.3.4 Gene expression analysis in the left ventricle of the heart

The expression of several genes with known implications in cardiac hypertrophy, fibrosis, calcium signaling, redox signaling and androgen-related genes was measured with RT-PCR.

3.3.4 Animal model and study design in paper IV

In paper IV, we used a transgenic mouse model of PCOS that overexpresses nerve growth factor selectively in the theca-interstitial cells of the ovary and they are termed 17NF. The 17NF mice were generated at the Oregon Health and Science University Transgenic/Gene Targeting Core, USA, by pronuclear microinjection of the transgene construct into fertilized eggs obtained from B6D2F1/J mice followed by transfer of the injected eggs into the uterus of a surrogate mother. The

transgene DNA construct is as follows: "The promoter sequence (17α-OH) was derived from the mouse gene [S41708], the source of the insulin II intron A was rat [J00748], the NGF minigene was human [V01511], and the GH polyadenylation signal was from rat [V01239]. All components except the promoter were part of a cassette known to express NGF" (62). The 17NF mice were bred to homozygosity. They were kindly provided to us by Dr. Cecilia Garcia-Rudaz, Monash University, Australia and were rederived at Comparative Medicine, Karolinska Institutet before experimental use. We used various batches of the homozygous 17NF mice for the developmental, reproductive and metabolic assessments, and we confirmed the transgene expression in each of them by genotyping. In all experiments, mice of B6D2F1 background were used as controls. Finally, mice were housed under a 12 h light–dark cycle, with controlled conditions and had free access to water and in-house chow food.

3.3.5   Methods used in Paper IV

3.3.5.1 Embryonic assessment

To investigate the impact of ovarian overexpression of NGF on fetal development, control and transgenic mice were mated and the embryonic development on gestational days 12.5 (GD12.5) and 18.5 (GD18.5) was assessed. We chose these two critical timepoints, as on GD12.5 the gonad differentiation has been completed and on GD18.5 the gestational period is almost complete (expected delivery from day 19). To evaluate the development of the fetuses, the crown to rump length and gonad size were measured. Proper function of the placenta is critical for the development of the embryo; therefore, we also assessed the placental morphology and target genes that regulate fetal growth and placental function.

3.3.5.2 Reproductive assessment

The 17NF mice have compromised reproductive activity (62). To further investigate this finding and assess whether it has embryonic origins, we measured the number of germ cells in the developing fetuses on GD12.5 and GD18.5. Germ cells were immunostained and distinguished by mouse vasa homologue or E-cadherin staining. Moreover, primary oocyte maturation was assessed by immunostaining in GD18.5 ovaries.

In 3-week-old pups, anogenital distance was measured as an indicator of *in utero* androgen exposure, and the estrous cyclicity was assessed in adult mice, as described in Part I (Paper I).

3.3.5.3 Metabolic assessment

In vivo testing: The body weight development was followed from adolescence to adulthood. In adult mice, body fat and lean mass were measured by echoMRI, and

glucose metabolism was assessed by an oral glucose tolerance test and an insulin tolerance test. Moreover, mice were placed in metabolic cages to assess energy metabolism by indirect calorimetry.

In vitro and ex vivo testing: In addition to the *in vivo* testing of glucose metabolism, we also investigated the glucose uptake in target tissues using ^{14}C radiolabeled glucose (D-[^{14}C(U)]-Glucose- tracer). This glucose uptake assay allows the measure of glucose uptake and glucose transport (incorporation into lipids).

We isolated adipose precursor cells from the stromal vascular fraction of subcutaneous fat depots from 6-week-old mice. Adipose precursor cells were then differentiated to mature adipocytes, and expression of key genes involved in the differentiation process were assessed during three timepoints. The first timepoint was the start of the differentiation process, followed by the 6th day of the differentiation in which preadipocytes have been formed and completed by the 12th day, in which mature adipose cells have been formed.

Ex-vivo lipolytic rate was measured in subcutaneous and parametrial fat explants from adult mice. Here we measured both basal lipolysis (no stimulation) and stimulated lipolysis (stimulation with forskolin). Also, morphological assessment of these two white fat explants was performed by histological examination.

Changes in the lipolytic activity in the fat tissue can lead to increased free fatty acid release into the circulation and to increased triglyceride content in the liver. Therefore, we measured the triglyceride and non-esterified fatty levels in the serum, and triglyceride deposition in the liver by *in vitro* enzymatic colorimetric methods.

3.3.5.4 *Plasma catecholamines*

Plasma catecholamine levels were determined with a validated high-pressure liquid chromatography – electrochemical detection (HPLC-EC) system.

3.3.5.5 *Gene expression in target tissues*

The expression of target genes in the embryonic ovaries, adult ovaries, placenta, white adipose tissue and liver were analyzed by RT-PCR.

3.3.6 Statistical analysis in Papers III and IV

The statistical analyses were performed using the SPSS software in Paper III and the SPSS software and GraphPad in Paper IV. One-way ANOVA or two-way ANOVA or Student's t-test was used in Paper I, and Student's t-test was used in Paper IV in normalized data. The detailed description of the statistical tests used in each experiment, can be found in the respective papers. The α level was set at $P \leq 0.05$

3.4 Results of studies III and IV

3.4.1 Paper III: Maternal androgen excess induces cardiac hypertrophy and left ventricular dysfunction in female mice offspring

Here we showed that maternal androgen excess, with or without maternal obesity, leads to cardiac hypertrophy in the adult female offspring, as indicated by increased interventricular septal and posterior wall thickness. These structural cardiac changes were accompanied by expression changes of genes involved in pathological cardiac hypertrophy (*Nppb, Trim63*), fibrosis (*Tgfbeta1, Col1a1*), calcium signaling (*Ryr2, Atp2a2*), redox signaling (*Sod2*) and steroid metabolism (*Srd5a2, Srd5a3)*. These cardiac changes were not associated to metabolic dysfunction or changes in blood pressure in the adult mice. We investigated the neonatal female pups for early cardiac changes, which could account for the long-lasting effects observed in adulthood. Interestingly, we found that the left ventricle from neonatal PNA mice had upregulated expression of genes involved in cardiac hypertrophic remodeling (*Mef2c*), calcium signaling (*Slc8a2*), and the androgen-related gene, *Odc1*, along with downregulation of the *Nox4* gene that is involved in redox signaling. These findings indicate that the cardiac changes in adult mice are a result of early cardiac remodeling due to *in utero* exposure to androgens. Further, by using another mouse model of PCOS with continuous exposure to androgens from adolescence to adulthood, we showed that continuous exposure to androgens leads to cardiac hypertrophy, similarly to the PNA model. Moreover, these mice had impaired vascular function, as indicated by increased contractile responses of the mesenteric arteries. Treatment with the antiandrogen flutamide partially alleviated these effects.

3.4.2 Paper IV: Excess of ovarian nerve growth factor impairs embryonic development and causes reproductive and metabolic dysfunction in adult mice

In this study we used a transgenic mouse model of PCOS (17NF mice), and we showed that ovarian NGF excess can lead to adverse fetal development and to reproductive and metabolic dysfunction in adult life. Starting with the embryonic assessment, we found that the 17NF female mice were growth-restricted, partly due to placenta dysfunction. Moreover, we showed that they had a reduced number of germ cells and delayed gonocyte and primary oocyte maturation. In adult life, they displayed disrupted estrous cyclicity accompanied by ovarian expression changes of steroidogenic genes and epigenetic markers. The adult 17NF mice had higher circulating levels of dopamine. Assessment of their metabolic profile revealed an increased fat mass, impaired glucose tolerance and lower energy expenditure. In addition, we found a larger volume of the subcutaneous fat depot with enlarged

adipocyte size in the 17NF mice, accompanied by dysregulated gene expression (*Ppardelta*, *Zfp423*, *Cebpbeta*, and *Fasn*), and an enhanced preadipocyte differentiation. In the parametrial fat of the 17NF, we demonstrated a heterogeneous distribution of adipocytes with a mixture of smaller, immature cells and larger mature adipocytes, and dysregulated expression of genes involved in adipogenesis (*Ppargamma*), glucose metabolism (*Slc2a4*), catecholamine metabolism (*Adrb3*, *Comt*, *Maoa*), fibrosis (*Col6a1*, *Col6a3*) and inflammation (*Tnf*). The 17NF mice also developed liver steatosis. Finally, we showed an increased glucose uptake in the ovary and brown adipose tissue, and reduced glucose uptake in the extensor digitorum longus muscle.

3.5   Conclusions

In this part of the thesis, I described the cardiovascular and metabolic complications that are often present in women with PCOS. The developmental origins of these complications were investigated by using three different mouse models of PCOS. In both studies we focused on the effects of an adverse maternal and/or postnatal environment on female health. In paper III, by using the PNA model, we demonstrated an early cardiac remodeling that led to cardiac hypertrophy in adulthood without the presence of other metabolic complications. We also showed that postnatal exposure to androgens led to cardiac hypertrophy and increased vasocontractile responses. In paper IV, by using the transgenic 17NF mice, we demonstrated that excess of ovarian NGF accompanied by ovarian sympathetic hyperactivity, can initiate disturbances in the developing fetus and further contribute to reproductive and metabolic dysfunction in adult life, affecting multiple organs. The cardiac profile of the 17NF mice warrants future investigation.

Our findings highlight the complexity of the syndrome and pinpoint that different stimuli can initiate the PCOS-associated symptoms, at least in our preclinical models. To identify the origins of these stimuli, the maternal androgen excess (Paper III) and ovarian NGF excess (Paper IV), was out of the scope of these studies, but they are fundamental questions to be answered. To date, there are no naturally occurring animal models of PCOS, with the exemption of a study that identified hyperandrogenic rhesus monkeys that developed PCOS-related traits (167). The animal models of PCOS that we are currently using provide valuable information about the order of the events that take place and how common features of the syndrome affect the manifestation of others, but they cannot identify the genesis of PCOS.

4   THIS THESIS AND FUTURE DIRECTIONS

It is becoming evident from the current work that PCOS is a complex syndrome, which affects multiple organs and may have strong environmental and epigenetic influences, in addition to its multigenic character.

Here, we focused on the environmental factors that can contribute to the onset of the syndrome. We and others have provided adequate evidence to support that maternal androgen excess can initiate PCOS-like features in the offspring for several generations, i.e. transgenerational transmission. Future studies may focus on the molecular mechanisms that govern these phenotypic manifestations. Moreover, due to the multi-organ involvement, studies using advanced methods that allow the study of multi-organ communication would provide novel knowledge about the interaction between target organs and would possibly help to identify common affected pathways with ultimate goal the development of more effective treatments for women with the syndrome. This approach could be implemented using even human material (e.g. cells) to provide direct translational outcomes.

Future considerations for the studies in the current thesis:

Studies I and II: The evident sexually dimorphic behavior in both the animal and the human studies points towards the need to study further the involved mechanisms. Some mental health disorders are more prevalent in females, while others in males. The exact mechanisms of action are not clear. A better understanding of the sex steroid effects in the male and female brain neural systems could help to the development of sex-oriented treatments for more effective drug responses. In this section, we also demonstrated that the anxiety-like behavior can be transmitted transgenerationally. The epigenetic mechanisms that drive this transmission are unknown and need to be studied.

Last, but not least, a few register-based studies indicate that mental health disorders in the offspring of women with PCOS may be both due to maternal androgen excess and genetic predisposition. Both factors are important, and it is critical to identify the genes that may drive the increased risk for mental disorders, and whether these genes are shared between PCOS and specific mental disorders. Regarding maternal androgen excess, pharmacological and/or lifestyle interventions aiming to improve androgen levels in these women could bring substantial benefits to their children and future studies should assess the effects of different maternal treatment options on offspring health.
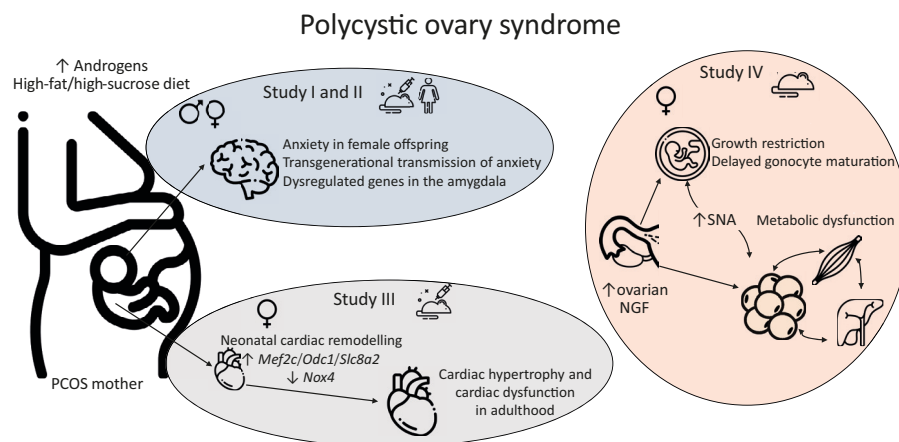
Studies III and IV: The results of **study III** concerning the cardiovascular risk in women with the syndrome and their daughters need further investigation. Despite the increasing number of studies showing adverse cardiac manifestations in both populations, little is known about the clinical importance of these data, and whether they can lead to an increased risk of mortality later in life. A limited number of

studies have assessed the mortality rates in these women and the results indicate a similar prevalence of adverse cardiovascular events, despite the increased cardio-vascular risk factors in women with PCOS (168-170). Therefore, studies including a larger sample size and broader age ranges would provide a more complete picture.

Flutamide was used in study III to prevent the possible adverse effects of andro-gens on the cardiovascular system. In our study, it did not effectively alleviate the increased vasoconstriction after androgen exposure. Considering the results of our study, future studies should investigate the efficacy of various doses of flutamide treatment on the cardiovascular system and compare flutamide to other anti-androgenic compounds that reduce the 5a-reductase activity, such as finasteride. Both flutamide and finasteride have been used as a treatment to the androgen-related symptoms (e.g. hirsutism) in women with PCOS (165).

Study IV provided indication that overexpression of ovarian NGF, accompanied by an increase in ovarian sympathetic activity, can initiate many of the symptoms observed in women with PCOS. Women with PCOS often deliver babies small or large for gestational weight (171, 172). Whether this is an outcome of changes in sympathetic nerve activity needs further research. We also observed reproductive and metabolic dysfunction in adult mice. These findings recapitulate many aspects of the human PCOS condition. Despite the fact that we provided an extensive picture of the adverse effects that take place in these mice, the exact sequence of events is not known. Moreover, in future studies it would be interesting to inves-tigate the sympathetic nerve activity in daughters of women with the syndrome, and the possible association between changes in sympathetic nerve activity and adverse phenotypic manifestations. Finally, studies modulating sympathetic nerve activity in women with PCOS could provide important insight into the possible effects on the PCOS-symptoms.

Graphical summary of this thesis:

Polycystic ovary syndrome

The icons used in this figure were made by https://www.flaticon.com/authors/freepik.

5 ACKNOWLEDGEMENTS

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