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POLYCYSTIC OVARY SYNDROME (PCOS): ROLE OF ANDROGENS AND OBESITY ON PLACENTAL FUNCTION AND FETAL DEVELOPMENT

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Polycystic Ovary Syndrome (PCOS): Role of androgens and obesity on placental function and fetal development

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Para mi hermosa familia

ABSTRACT

Polycystic ovary syndrome (PCOS) is a common endocrine and metabolic disorder affecting women in childbearing age with a prevalence of up to 17.8%. The syndrome is characterized by hyperandrogenism, irregular cycles and polycystic ovaries. The etiology of PCOS is unclear, but it is thought to be multifactorial. There is a strong association between hyperinsulinemia and hyperandrogenism in PCOS, but the mechanisms behind their relationship with PCOS are not fully understood. Obesity and an aberrant metabolic profile is common in women with PCOS, and 50-70% of them are insulin resistant, which increase the risk of developing type 2 diabetes (T2D), independently of body mass index (BMI) and age.

Women with PCOS have a reduced fertility rate, and when they become pregnant either by natural process or by assisted reproduction techniques, they are at higher risk of developing pregnancy complications including preeclampsia and gestational diabetes that worsen the prognosis of their own health and the health of the fetus. It is not well known if and how the intrauterine environment affects the fetus. In women with PCOS, a potential effect on the fetus can be hypothetically driven by direct exposure of high maternal androgens to the fetus or via dysregulation of placenta function.

In both non-pregnant and pregnant women with PCOS, lifestyle modification including diet and physical exercise is the first line treatment. However, scarce information about treatment of pregnant women with PCOS to prevent the adverse outcomes is found in the literature. Acupuncture has been proposed as one treatment as has been shown to increase uterine artery blood flow in non-pregnant women. Importantly, acupuncture is usually reported to have less negative side-effects than pharmacological strategies.

The overall aim of this thesis was to determine the role of androgens and obesity in pregnancy and whether acupuncture could modulate placenta function and fetal growth. First, in a cross-sectional study, maternal blood and placental tissue were collected at delivery from 38 women with PCOS without pregnancy complications and 40 control pregnant women to investigate signal transducer and activator of transcription 3 (STAT-3) and mechanistic target of rapamycin (mTOR) signaling pathways in placenta. Second, in rats with prenatal androgenization (PNA), we evaluated markers of steroidogenesis, angiogenesis and sympathetic activity, and we tested the hypothesis that acupuncture with low-frequency electrical stimulation prevents any alteration in the expression of those markers. Thirdly, as women with PCOS are often overweight or obese, we investigated maternal growth and metabolism, placenta weight, placenta steroid receptor expression, and liver fat content from mice exposed to maternal androgen excess with or without diet-induced maternal obesity. Moreover, we performed a global proteomic analysis in placenta and fetal liver to find novel molecules that could be involved in the observed alterations.

Pregnant women with PCOS display abnormal steroidogenic state, altered placenta gene expression of steroidogenic enzymes and molecules related to fetal growth as determined by the activation of STAT-3. Moreover, diet-induced maternal obesity and maternal androgen excess induced hepatic triglyceride accumulation and dysregulation of de novo lipogenesis in

mothers. In proteomic analysis of placenta and fetal liver, we found a novel Catechol-O-Methyltransferase (COMT) phosphorylation that was common in fetal liver and placenta. We also found altered gene expression of enzymes in the liver of female offspring suggesting that the sympathetic nervous system could play a role in the metabolic, reproductive or behavioral disturbances known in offspring of PCOS. These observations are supported by the finding that electroacupuncture given to pregnant dams exposed to testosterone increased systolic blood pressure, decreased fetal and placental growth and altered the expression of markers of angiogenesis, indicating an increased sympathetic nervous activity, contrary to our hypothesis.

Besides pregnancy complications, it seems that molecular signatures might make women with PCOS more sensitive and vulnerable to metabolic challenges, which potentially can explain long-term health consequences in their offspring. Moreover, it seems that the sympathetic nervous system plays an important role for fetal development in androgenized dams.

LIST OF SCIENTIFIC PAPERS

I. Placental STAT3 signaling is activated in women with polycystic ovary syndrome. Maliqueo M, Sundström Poromaa I, Vanky E, Fornes R, Benrick A, Åkerud H, Stridsklev S, Labrie F, Jansson T, Stener-Victorin E. Hum Reprod. 2015 Mar;30(3):692-700. doi: 10.1093/humrep/deu351. Epub 2015 Jan 20.

II. Maternal testosterone and placental function: Effect of electroacupuncture on placental expression of angiogenic markers and fetal growth. Fornes R, Hu M, Maliqueo M, Kokosar M, Benrick A, Carr D, Billig H, Jansson T, Manni L, Stener-Victorin E. Mol Cell Endocrinol. 2016 May 18;433:1-11. doi: 10.1016/j.mce.2016.05.014. [Epub ahead of print] PMID:27208621

III. The effect of androgen excess on maternal metabolism, placental function and fetal growth in obese dams. Fornes R, Maliqueo M, Hu M, Hadi L, Jimenez M, Ebefors K, Nyström J, Labrie F, Jansson T, Benrick A, Stener-Victorin E.. Sci Rep. 2017 Aug 14;7(1):8066. doi: 10.1038/s41598-017-08559-w.

IV. Proteomic analyses of placenta and fetal liver from mice with maternal androgen excess and diet-induced obesity. Fornes R, Manti M, Qi X, Vorontsov E, Sihlbom C, Nyström J, Sundström-Poromaa I, Jerlhag E, Maliqueo M, Hirschberg AL, Benrick A, Stener-Victorin E. (Manuscript)

SCIENTIFIC PAPERS NOT INCLUDED IN THIS THESIS

I. Maternal testosterone exposure increases anxiety-like behavior and impacts the limbic system in the offspring. Hu M, Richard JE, Maliqueo M, Kokosar M, Fornes R, Benrick A, Jansson T, Ohlsson C, Wu X, Skibicka KP, Stener-Victorin E. Proc Natl Acad Sci U S A. 2015 Nov 17;112(46):14348-53. doi: 10.1073/pnas.1507514112. Epub 2015 Nov 2

II. Epigenetic and Transcriptional Alterations in Human Adipose Tissue of Polycystic Ovary Syndrome. Kokosar M, Benrick A, Perfilyev A, Fornes R, Nilsson E, Maliqueo M, Behre CJ, Sazonova A, Ohlsson C, Ling C, Stener-Victorin E. Sci Rep. 2016 Mar 15;6:22883. doi: 10.1038/srep22883.

III. Tandem mass spectrometry determined maternal cortisone to cortisol ratio and psychiatric morbidity during pregnancy-interaction with birth weight. Hellgren C, Edvinsson Å, Olivier JD, Fornes R, Stener-Victorin E, Ubhayasekera SJ, Skalkidou A, Bergquist J, Sundström-Poromaa I. Psychoneuroendocrinology. 2016 Apr 7;69:142-149. doi: 10.1016/j.psyneuen.2016.04.006. [Epub ahead of print]

IV. Exercise differentially affects metabolic functions and white adipose tissue in female letrozole- and dihydrotestosterone-induced mouse models of polycystic ovary syndrome. Marcondes RR, Maliqueo M, Fornes R, Benrick A, Hu M, Ivarsson N, Carlström M, Cushman SW, Stenkula KG, Maciel GAR, Stener-Victorin E. Mol Cell Endocrinol. 2017 Jun 15;448:66-76. doi: 10.1016/j.mce.2017.03.025. Epub 2017 Mar 24.

CONTENTS

LIST OF ABBREVIATIONS

A4	Androstenedione
AKR1C3	Aldo-keto reductase family 1 member C3
AMH	Anti-Müllerian hormone
AR	Androgen receptor
BCA	Body composition analyzer
BMI	Body Mass Index
COMT	Catechol-O-methyl transferase
CYP17	Cytochrome P450 17
CYP19A1	Enzyme Aromatase
DEXA	Dual Energy X ray Absorptiometry
DHEA	Dehydroepiandrosterone
DHEAS	Dehydroepiandrosterone Sulfate
DHT	Dihydrotestosterone
EA	Electroacupuncture
FAI	Free Androgen Index
FFA	Free Fatty Acids
FSH	Follicle-stimulating hormone
HA	Hyperandrogenism
HOMA-IR	Homeostatic model assessment of Insulin Resistance
IGF1	Insulin-like growth factor 1
IGFBP1	IGF Binding Protein 1
IR	Insulin Resistance
LGA	Large for Gestational Age
LH	Luteinizing Hormone
MRI	Magnetic Resonance Imaging
mTOR	Mechanistic target of rapamycin

NGF	Nerve growth factor
NIH	National Institute of Health
OD	Ovarian dysfunction
OGTT	Oral Glucose Tolerance Test
PCOM	Polycystic ovarian morphology
PCOS	Polycystic ovary syndrome
SGA	Small for Gestational Age
SHBG	Sex Hormone-Binding Globulin
SNP	Single Nucleotide Polymorphism
STAT3	Signal transducer and activator of transcription 3
T2D	Type 2 diabetes
TG	Triglyceride

1 INTRODUCTION

1.1 POLYCYSTIC OVARY SYNDROME

Polycystic ovary syndrome (PCOS) is the most common endocrine and metabolic disorder in women [1], with a prevalence of up to 17.8% and is characterized by hyperandrogenism, irregular cycles and polycystic ovaries [2, 3]. Obesity and an aberrant metabolic profile are common in women with PCOS, and 50- 70% of them are insulin resistant [4, 5]. Most women with PCOS are able to compensate for their insulin resistance (IR), but a large proportion of them have altered beta-cell function [6-8] causing glucose intolerance, which increase the risk of developing type 2diabetes (T2D), independently of body mass index (BMI) and age [9]. Further, women with PCOS have an increased risk of developing dyslipidemia and hypertension [10, 11], with an increased prevalence of metabolic syndrome [12]. The etiology of PCOS is unclear, but it is thought to be multifactorial. There is a strong association between hyperinsulinemia and hyperandrogenism (HA) in PCOS, but the mechanisms behind their relationship with PCOS are not fully understood [13].

Most of the research within the field has been focused on how to improve the metabolic management of women with PCOS and to develop techniques that increase the fertility rate [14]. However, less attention has been paid to the pathophysiology involved in adverse pregnancy outcomes and the long-term health consequences in the offspring born to mothers with PCOS. Indeed, it is well known that , on one hand, women with PCOS are at increased risk of having pregnancy complications such as preeclampsia, gestational diabetes and preterm deliveries [15-18]. On the other hand, their newborns, are more often born small for gestational age (SGA) [15-17, 19] or large for gestational age (LGA) [18, 20] and have an increased risk of meconium aspiration, hospitalization in intensive unit care and perinatal mortality [18]. Further, daughters of women with PCOS in peripubertal stage exhibit features of abnormal reproductive development [21, 22] and an altered adrenal function [1]. Not only daughters of women with PCOS are affected, sons of women with PCOS also display some metabolic derangements during peripubertal age, likely because of the exposition to an adverse environment during fetal life [23].

1.1.1 Definition

PCOS is a complex entity that include a variety of signs such as clinical or biochemical hyperandrogenism, ovulatory dysfunction (oligo- or anovulation) and polycystic ovary morphology. Moreover, the diagnosis should be established in the absence of other diagnoses

like Cushing syndrome, congenital adrenal hyperplasia, androgen-producing tumors and hyperprolactinemia [24].

1.1.2 Diagnostic criteria

The first description of PCOS was done by Drs Stein and Leventhal in 1935 [25]. They described 7 cases where women had polycystic ovaries in association with amenorrhea and most of them displayed hirsutism [25, 26]. It was not until 1990 that the first diagnostic criteria was settled by experts from the National Institute of Health (NIH) in USA. The diagnostic criteria included hyperandrogenism and oligo/anovulation, excluding the polycystic ovarian morphology (PCOM) [27] (Table 1). In 2003, in the Rotterdam consensus conference, experts broaden the diagnostic criteria including the PCOM as a clinical sign to be considered together with the criteria determined by NIH consensus in 1990 [24] (Table 1). In 2006 the androgen excess and PCOS (AE-PCOS) society stated that androgen excess is the principal hallmark of the disorder, therefore, the diagnosis should be based on the presence of hyperandrogenism plus ovarian dysfunction (oligo-anovulation or PCOM) [28]. Twelve years later, in 2012, the Evidence-based Methodology Workshop on PCOS, sponsored by NIH decided to maintain the broader Rotterdam criteria together with the specification of the phenotypes as follow: 1) Clinical and/or biochemical hyperandrogenism (HA) + Ovulatory Dysfunction (OD), that include oligo- or anovulation; 2) HA + PCOM; 3) HA + OD + PCOM and 4) OD + PCOM [29] (Table 1). This phenotypic classification would improve research and clinical practice for patient management according to the risk of co-morbidities, such as metabolic syndrome, T2D and cardiovascular disorder, for instance.

Table 1. Diagnostic criteria since 1990.

	NIH 1990 [27]	ESHRE/ASRM (Rotterdam) 2003[24]	AE-PCOS 2006 [28]	NIH 2012 [29]
Findings	HA OA	HA OD PCOM	HA OD or PCOM	HA OD PCOM
Phenotypes	HA+OA	1) HA+OD+PCOM 2) HA+OD 3) HA+PCOM 4) OD+PCOM	1) HA+OD+PCOM 2) HA+OD 3) HA+PCOM	1) HA+OD+PCOM 2) HA+OD 3) HA+PCOM 4) OD+PCOM

HA: Hyperandrogenism, T >50 ng/dl (Measured by LC/MS-MS) or clinical signs (Acne, alopecia and or hirsutism; OA: Oligo/anovulation: cycles >35 days; OD: Ovarian dysfunction; cycles >35 days or progesterone levels ≥ 7.0 ng/mL; PCOM: Polycystic ovarian morphology, defined by 25 follicles measuring 2 to 9 mm in the whole ovary or ovarian size >10 mL [30].

Phenotypes 1 and 2 (classic forms of PCOS), are more prevalent and severe [27, 28, 31] and are associated with other pathological conditions such as insulin resistance and T2D, metabolic syndrome, obesity, atherogenic dyslipidemia and cardiovascular disease (CVD) [32]. Further, these phenotypes account for two-thirds of all women with PCOS [33].

1.1.3 Epidemiology of PCOS

The prevalence of PCOS varies depending on the diagnostic criteria used. While with the NIH criteria the prevalence is up to 6.1% and 8.7%, the prevalence with the Rotterdam criteria is up to 19.9% and 17.8% in the same populations [3, 34]. PCOS also has been shown to be more prevalent among women with type 1 diabetes mellitus [35], T2D [36], gestational diabetes [37] and obese patients [38], although the latter is more controversial [39].

In PCOS, the highest prevalences of obesity was reported in studies from United States (76%), and Australia (61%) while 15% and 19% of the population was overweight, respectively [40, 41]. However, the prevalence is different depending on whether the study population has been referred or is an unselected population indicating referral bias in the estimation of the prevalence of the obesity among women with PCOS. In United States, the prevalence of obesity in women with PCOS in a referral group has been shown to be 63% whereas in the unselected population the prevalence is 28.1%, similar to the unselected control (28.4%) [42]. In that study the severity of the syndrome was also different between study groups. While in the referral

PCOS group the most severe phenotype, e.g. oligo-anovulation + hirsutism + HA, was 52.7%, in the unselected group the phenotype was present only in 20.3% of the patients [42].

1.2 PATHOPHYSIOLOGY OF PCOS

Despite detrimental impact on women's health, the etiology of PCOS is not well understood. The pathophysiology encompasses signs and symptoms that originate from different key phenomenon including steroidogenic, metabolic and genetic abnormalities resulting in a broad spectrum of the disorder with mild and severe presentation where the hyperandrogenism is the hallmark of the syndrome (Fig.1).

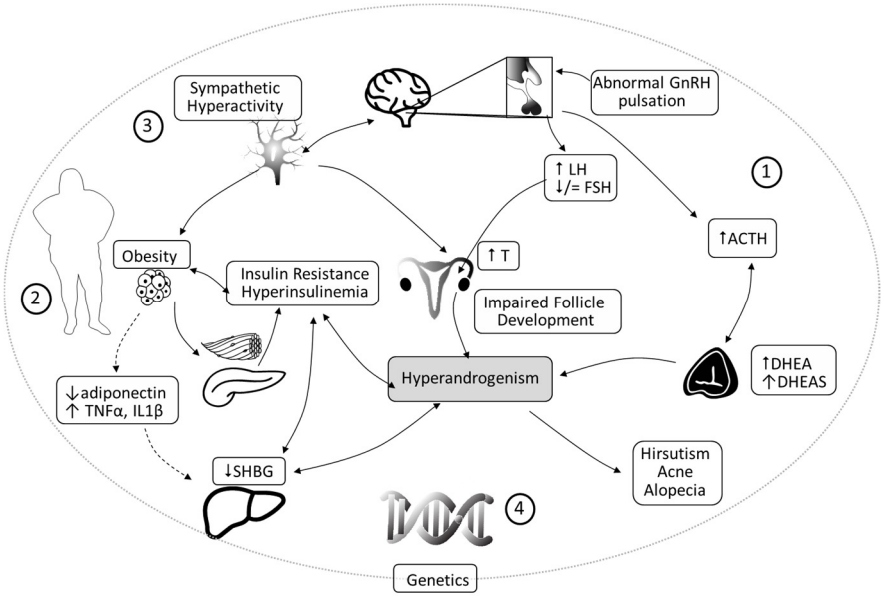


Figure 1. *Pathophysiology of PCOS.*

1. *Increase in LH pulse frequency and amplitude with normal or low FSH secretion results in an elevated LH/FSH ratio [43]. The unopposed LH secretion disrupts follicle development and results in thecal hyperplasia contributing to enhanced androgen production, and follicular atresia. Moreover, an increased hypotalamic-pituitary adrenal activity increase the secretion of DHEA and DHEAS that contribute to hyperandrogenism.*
2. *Hyperinsulinemia as a compensatory response to reduced peripheral insulin insensitivity [44]. Insulin and in a minor degree IGF-I stimulate the androgen production by theca and stromal cells Obesity with a predominant abdominal fat accumulation which has been related to higher levels of testosterone and decreased SHBG secretion [45].Obesity decreases anti-inflammatory cytokines and induces pro-inflammatory cytokines, that in turn decrease synthesis of SHBG increasing the free androgen availability*
3. *Increased sympathethic nerve activity that is related to higher androgen levels, obesity and insulin resistance*
4. *Genetic component of the development of PCOS.*
   LH: luteinizing hormone;FSH: follicle stimulating hormone; DHEA: Dehydroepiandrosterone; DHEAS Dehydroepiandrosterone sulfate; IGF-I: Insulin-like growth factor 1.

1.2.1 Androgen excess

Normally, ovaries and adrenal glands are under control of luteinizing hormone (LH) and adrenocorticotrophic hormone (ACTH), respectively, and contribute to the synthesis of sex steroids [46-48]. In PCOS, a consistent elevation of GnRH pulse frequency cause an increase in LH pulse frequency and amplitude with normal or low follicle stimulating hormone (FSH) secretion and results in an elevated LH/FSH ratio [43]. In the normal ovary, LH stimulate the theca cells to synthetize androgens which in turn are converted to estrogen by CYP19A1 (or P450aromatase), in granulosa cells [49] (Fig. 2). In PCOS, activity of enzyme Cytochrome P450 c17 (CYP17), which converts progesterone to 17-hydroxyprogesterone and from 17-hydroxyprogesterone to androstenedione (A4) is exaggerated and a decreased activity of CYP19A1 favours androgen production on these women [50]. Moreover, the unopposed LH secretion increases the synthesis of androgens by theca cells and results in thecal hyperplasia contributing to enhanced androgen production, disruption of follicle development and follicular atresia.

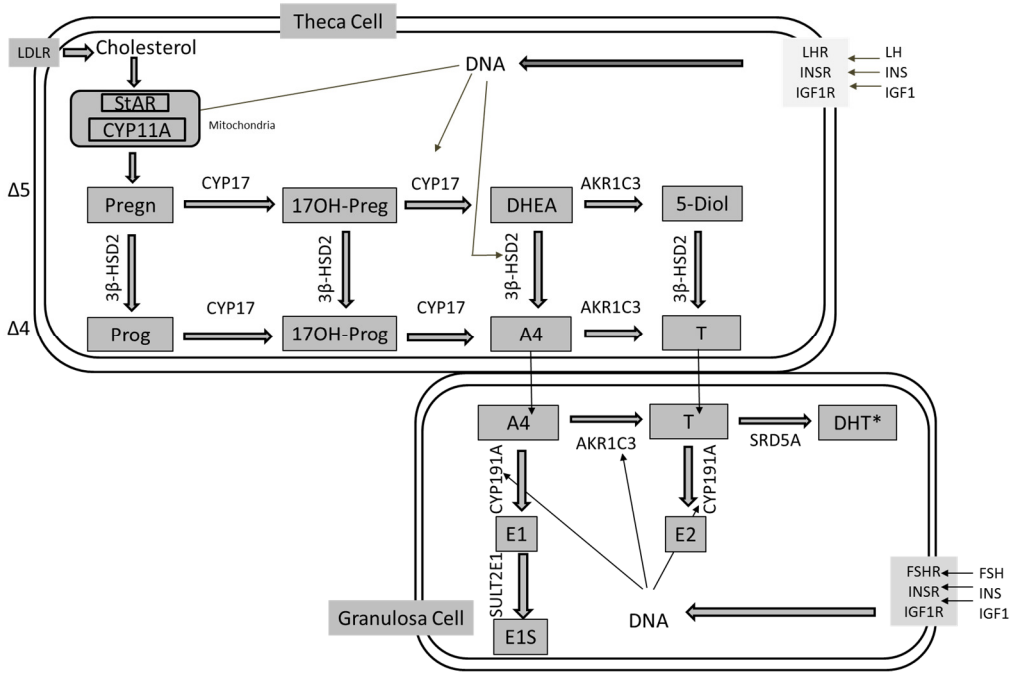


Figure 2. *Ovarian steroidogenesis. Granulosa and theca cells contribute to ovarian steroidogenesis. Theca cell contains enzymatic machinery for the synthesis of Androstenedione and Testosterone. Those steroids diffuse to granulosa cells for the conversion into estrogens. StAR: steroidogenic acute regulatory protein; CYP17: Cytochrome P450 c17; 3βHSD2: 3 beta-hydroxysteroid dehydrogenase/Delta 5-->4-isomerase type 2; AKR1C3: Aldo-keto reductase family 1 member C3 (also known as 17-beta-HSD 5); CYP11A1; cytochrome P450 family 11 subfamily A member 1 (or cholesterol side chain cleavage cytochrome P450); CYP19A1: Cytochrome P450 19A1(or P450Aromatase); SDR5A: 3-oxo-5-alpha-steroid 4-dehydrogenase 1 (or 5α-reductase). * , conversion mostly in peripheral tissues.*

On the other hand, the adrenal gland also contributes to the hyperandrogenism in women with PCOS. Normally, the adrenal cortex produces, in descending order, dehydroepiandrosterone (DHEA), A4 and in minor extent testosterone (T) throughout mainly the Δ^5 steroidogenic pathway [51] (Fig. 3). DHEA could be sulfated through DHEA sulfotransferase and released to the circulation as DHEA sulfate (DHEAS) [52]. These adrenal precursor androgens function as pre-hormones contributing in a large extent to the amount of the more potent androgens, T and dihydrotestosterone (DHT). For instance, 50% of the available T comes from the conversion of A4 in the liver, another 25% is synthetized directly in the adrenal and the rest in the ovaries [53]. DHEA is converted to A4 in the liver and directly to DHT from A4 in some peripheral tissues, without previous T formation. 50% of A4 and 20-30% of DHEA are produced in the ovaries and almost all the circulating DHEAS is produced in the adrenal cortex. Because of that, DHEAS is the best marker of adrenal androgen precursor production.

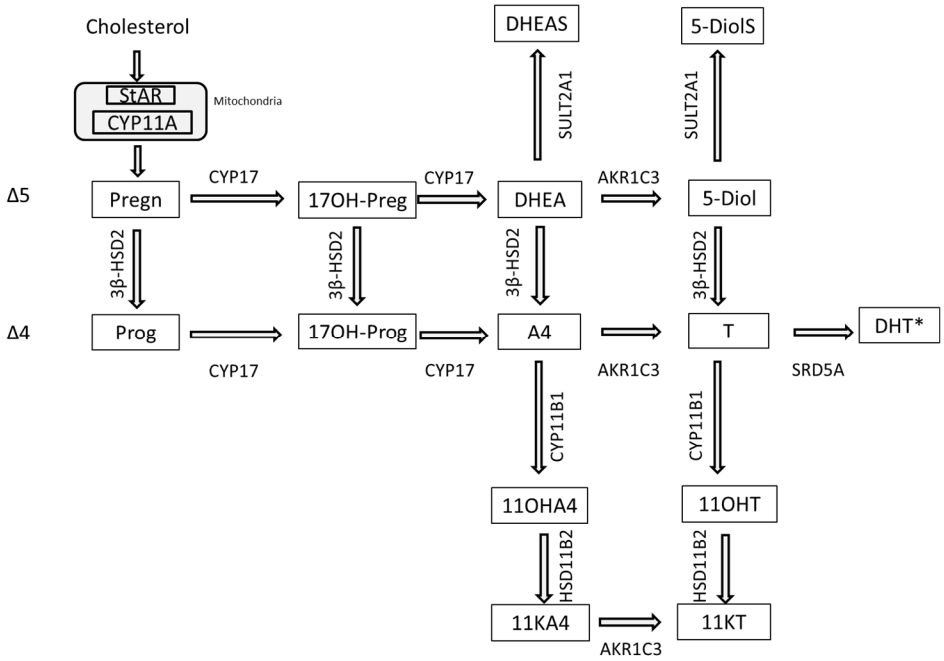


Figure 3. *Adrenal steroidogenesis. StAR: steroidogenic acute regulatory protein; CYP17: Cytochrome P450 c17; 3βHSD2: 3 beta-hydroxysteroid dehydrogenase/Delta 5-->4-isomerase type 2; AKR1C3: Aldo-keto reductase family 1 member C3 (also known as 17-beta-HSD 5); CYP11A1; cytochrome P450 family 11 subfamily A member 1 (or cholesterol side chain cleavage cytochrome P450); SDR5A: 3-oxo-5-alpha-steroid 4-dehydrogenase 1 (or 5α-reductase);CYP11B1: cytochrome P450 family 11 subfamily B member 1;HSD11B2: hydroxysteroid 11-beta dehydrogenase 2; SULT2A1:* sulfotransferase family 2A member 1*, *conversion mostly in peripheral tissues.*

In PCOS, 20-30% have androgen excess of adrenal origin [54]. Of note, DHEAS is decreased in relation to increased age. DHEAS is 20-30% higher in women with PCOS when adjusted

for age and race [52]. Interestingly, higher levels of androgen precursors, DHEA, DHEAS and A4 have been found in non-obese patients with PCOS compared to obese women with PCOS [55]. Moreover, adrenal hyperandrogenism in women with PCOS has been associated with reduced insulin sensitivity and increased blood pressure [56].

Insulin resistance with compensatory hyperinsulinemia is common in PCOS [44]. Insulin increase the activity of CYP17 that also favors the conversion of progestogen precursors to androgen production in adrenals and ovaries [51]. A recent study shows that obesity-induced hyperandrogenic anovulation is associated with 20 times higher levels of insulin and is reversed in transgenic littermates mice lacking the insulin receptor in theca cell [57]. The bioavailability of androgens is also favored because insulin decreases the synthesis of sex hormone-binding globulin (SHBG) and insulin-like growth factor binding protein (IGFBP-1) by the liver. This in turn increase free androgen availability and IGF concentration in the ovary, stimulating the production of androgen via stimulation of IGF receptors.

Another factor that could increase the androgen excess of ovarian origin is anti-müllerian hormone (AMH). AMH is synthetized by granulosa cells of primary, pre-antral and small antral follicles [58]. Of note, primordial follicles do not produce AMH [59]. Normally, AMH regulates folliculogenesis by the inhibition of primordial follicle recruitment from the resting pool in order to select for the dominant follicle, and decreasing the sensitivity of small antral follicles to FSH activity after which the production of AMH diminishes [60]. In other words, AMH functions as a gatekeeper for the rate of depletion of primordial follicles and selection of maturing follicles [61]. AMH is significantly higher in women with PCOS due to an increased number of antral follicles and also a higher production per antral follicle, facilitating the follicular arrest in PCOS [61]. It is hypothesized that higher AMH in PCOS increase the follicular unresponsiveness to FSH and failure of follicular development lead by AMH-induced inhibition of aromatase, estradiol, and FSH and LH receptor acquisition in granulosa cells [61].

PCOS does not seems to have a Mendelian pattern of inheritance. Many studies have shown a large list of genes [62-64] that are related to the bioavailability and synthesis of androgen (*CYP17A1, CYP19, AR, SHBG*), insulin signaling and metabolism (*INSR, IRS1, IRS2, PPARG, ADIPOQ*), folliculogenesis (*FSHR, LHCGR* and *AMHR*) and inflammation (*IL1A, IL1B, IL6, IL18, PAI1, FBN3, TNF*), among others [62, 64]. Genome-Wide Association Studies (GWAS) executed in Han Chinese women [65] and later replication in European population [66] demonstrated around 20 single nucleotide polymorphisms (SNPs) associated with PCOS. Among them were *THADA* and *DENND1A* genes. The overexpression of a splicing variant, DENND1A.V2 in theca cells increase the *CYP17A1* and *CYP11A1* gene expression and

androgen biosynthesis. Although the mechanism is still unknown, studies show that a genetic component is also part of the pathophysiology of the syndrome [49, 62].

Although obesity is not part of the diagnosis, its presence exacerbates the PCOS phenotype [5]. Obesity with a predominant abdominal fat accumulation has been related to higher levels of testosterone and decreased SHBG secretion [45]. The enzyme aldoketoreductase type 1C3 (AKR1C3), which converts A4 to the biologically active androgen testosterone and is modulated by insulin [67], is abundantly expressed in subcutaneous fat from both women with simple obesity [68] and in women with PCOS, who also display a lower gene expression of CYP19A1 [69]. Of note, an *in vitro* study showed that insulin increases adipose tissue expression and activity of AKR1C3, whereas androgen exposure increases adipocyte *de novo* lipid synthesis which was reversed by pharmacologic AKR1C3 inhibition [70]

Adiponectin , an hormone considered as insulin sensitizer [71], has also been related to the pathophysiology of PCOS. The adiponectin level is decreased in obese PCOS patients compared to weight matched controls [72], and a defective secretion of adiponectin due to androgen excess has been reported. Further, adipocyte size together with circulating adiponectin and waist circumference has been demonstrated to be the strongest predictor for insulin resistance in women with PCOS [73]. Further, overexpression of adiponectin protects mice from developing a PCOS like metabolic, but not reproductive phenotype when exposed to DHT [74]. Comin *et al* has recently shown that adiponectin receptors (AdipoR1 and AdipoR2) are decreased in ovaries from women with PCOS, independent of BMI [75], suggesting an impaired adiponectin signaling.

1.2.2 Obesity and metabolic disturbances associated with PCOS

It is well accepted that obesity exacerbates the symptoms of PCOS, but there are controversies about whether obesity causes the syndrome. Obesity steadily increase in the developed world. In fact, the worldwide prevalence of obesity more than doubled between 1980 and 2014. In 2014, 40% of women aged 18 years and over were overweight [76].

The effect of obesity in the development of PCOS is related to insulin resistance and compensatory hyperinsulinemia. Insulin resistance range from 14 to 43% among women with PCOS and depend on the diagnostic criteria used. High BMI is also associated with lower SHBG levels, due to the fact that insulin suppresses the SHBG production from the liver [77]. Nevertheless, new insights have proposed that cytokines and adipokines, more than insulin, could have a role in the regulation of SHBG. Pro-inflammatory cytokines such as TNFα and interleukin 1β, downregulate the expression of SHBG in hepatocytes through down regulation

of hepatocyte nuclear factor 4 alpha (HNF4A) [78]. Further, adiponectin increases synthesis of SHBG via downregulation of genes related with *de novo* lipogenesis and increases the expression of those involved in fatty acid oxidation in HepG2 cells [78].

Studies have demonstrated that women with PCOS have increased abdominal or visceral fat accumulation [79]. However, the use of a gold standard method, magnetic resonance imaging (MRI) in age and weight matched case-controls does not demonstrate increased visceral fat content [73]. Moreover, in lean women with PCOS evaluated with MRI, the researchers found less visceral fat than control women without PCOS [80]. A recent study shows that alterations of lipid metabolism in women with PCOS persist after correction of central adiposity, with the worst metabolic profile associated with highest waist circumference (>0.98) [81]. Despite no clear evidence of more abdominal/visceral fat accumulation in women with PCOS, the adipose tissue function and morphology is hampered. Visceral fat has an increased lipolytic activity in response to catecholamines that increase the release of free fatty acids (FFA) to the liver trough portal circulation causing hepatic lipotoxicity and insulin resistance [82, 83]. Testosterone could have a role in visceral fat accumulation as demonstrated in iatrogenic hyperandrogenism in female-to- male transsexuals who were exposed to testosterone [84]. Rodent models also support a direct role for androgen excess in the accumulation of abdominal fat [74].

1.3 PREGNANCY AND PCOS

As stated before, pregnant women with PCOS display an increased prevalence of gestational diabetes, preeclampsia and preterm deliveries [15-18]. Their newborns show more often altered size and admissions to intensive care unit [15-20].

Pregnant women with and without PCOS have increased circulating estrogen and progesterone levels together with a decreased level of SHBG in the second trimester. Further, the level of DHEAS and free androgen index (FAI) is higher in pregnant women with PCOS compared with control women in the second trimester [85]. Moreover, insulin secretion in response to an oral glucose tolerance test (OGTT) is higher in the second trimester. Metabolic disturbances are worse in women with a hyperandrogenic phenotype [16, 86].

Women with PCOS show alterations in uterine artery Doppler indices in the first trimester of pregnancy, which could explain the increased risk for adverse perinatal outcome observed in women with the syndrome [87]. Moreover, the placenta displays higher rates of chorionic villitis and intervillositis in early pregnancy being more frequent in the more severe phenotypes (full-blown and non-PCO) than in the ovulatory and non-hyperandrogenic phenotypes [88]. At term, placenta from women with PCOS even without maternal complications, show more

macroscopic lesions than the placenta from control women [89]. Some studies have shown that women with PCOS have elevated blood pressure, even independent of BMI throughout the pregnancy [90], as well as altered uterine artery blood flow in the uterine artery, which is related to poor outcomes in pregnancy [16]. Thus, vascular alterations developed in pregnancy may affect placenta function as discussed before. Studies in women with preeclampsia, where fetal growth restriction (FGR) is more frequent, show altered placentation that could compromise the exchange of nutrients between mother and fetus [88].

1.4 PREGNANCY AND OBESITY

Around 28% [91] to 63.7% [42] of women with PCOS are obese, which may increase the risk of poor pregnancy outcomes commonly seen in women with the syndrome. Obese women are more likely to suffer from infertility and impaired fecundity, which suggest that the uterine receptivity is altered [92]. During pregnancy, obesity per se is related to adverse obstetric outcomes regardless if the obesity is established in the pre-pregnancy period [93] or due to an excessive weight gain during pregnancy. Obese women display lower amount of mRNA of the leptin receptor in the syncytio-trophoblast without increased leptin protein levels [94], suggesting the existence of leptin resistance as found in obesity [95]. Further, during pregnancy obese women have higher serum leptin than their non-overweight counterparts although a lower leptin levels per unit mass of adipose in overweight/obese women through the progression of the pregnancy has been reported [96].

Another hormone secreted by the adipose tissue that has an important role in pregnancy is adiponectin. Maternal adipose tissue is the primary source of circulating adiponectin during pregnancy and the synthesis in placenta at term has been questionable [97, 98]. Regardless of origin, adiponectin has been associated with the metabolic state during pregnancy and overweight/obese women who subsequently develop gestational diabetes have lower adiponectin than an euglycemic group during pregnancy [99]. Of note, the adiponectin/leptin ratio inversely correlates with homeostatic model assessment of Insulin Resistance (HOMA-IR) in pregnancies affected with insulin resistance [100].

Animal models aiming to mimic maternal obesity before and during pregnancy induce maternal hypertension and glucose intolerance [101], increase [102] or decrease [103] fetal weight, and enhance the stress in the adult offspring [104]. Further, obesity is associated with changes in the methylation profile of oocytes and liver from offspring predisposing development of metabolic disturbances [105]. All these effects could be accompanied by an altered placental function [106]. Indeed, in rats, obesity impairs mitochondrial dynamics in placenta and liver

from offspring, increasing the risk of being obese in adulthood [107]. Interestingly, fetal abnormalities such as fetal growth retardation and brain abnormalities are associated with a failure in oocytes more than an adverse intrauterine environment in mothers fed with high fat diet [103].

1.5 FETAL PROGRAMMING

During the last century, the idea that the placenta was the perfect filter that gives protection to the developing fetus has changed radically. A number of examples demonstrate that the intrauterine environment is not a closed and unmodifiable system, and that the placenta is not always a permeable barrier that ensure a healthy fetal development. During the Dutch famine in winter of 1944-1945, the population, including pregnant women, were under a caloric restriction up to 500 kcal/day. Higher rate of obesity in sons that were exposed to maternal caloric restriction in the first trimester was observed compared to those exposed in the second and third trimester [108]. The latent effects of an adverse intrauterine exposure was better developed by David Barker [109] who describe several ideas in his theory of origin of the diseases such as the fetal conditions are persistent and their effects in health could remain latent after several years [110]. This "memory" could be originated in a biological mechanism called "programming" earlier in life [111]. Indeed, Barker state that the undernutrition or inadequate oxygen supply during pregnancy would "change or program the physiology and structure of the body" [109]. Although not exempt of criticism, because of the low quality of the statistical analysis done by Barker, his disease origins idea has been developed and tested by thousands of scientists worldwide [112-115].

Androgens and obesity also program the fetus to develop metabolic [21] and reproductive dysfunction [1], and behavioral disorders [116] in their infancy, puberty or in the adulthood. Daughters from women with PCOS exhibit features of abnormal reproductive development with increased ovarian volume, and in later puberty increased levels of fasting and 2 h insulin, triglycerides, testosterone and low SHBG compared with control girls [21, 22]. A similar profile is also evident in older age in spite of having better reproductive profile [117]. Furthermore, at the age of 15, between 28-36% of adolescent daughters born to women with PCOS have irregular periods, clinical and biochemical hyperandrogenism and PCO morphology [118]. In more recent studies, the levels of androgens in mixed arterial and venous blood has shown different results with either low [19, 20] or high androgen levels compared with controls. Interestingly, one study demonstrated that the maternal androgen levels were slightly inversely correlated with the weight of the newborn [19]. Nevertheless, the measurement of the steroids was done by chemiluminescent immunoassays, which is known

to have a low sensitivity and specificity compared with e.g. liquid or gas chromatography mass spectrometry [30].

It is thought that the human placenta protects the fetus against hyperandrogenemia because placenta expresses CYP19A1, an enzyme that converts testosterone into estrogens [119]. Despite of that, it is not clearly established that androgens cross the placenta and affect the developing fetus. Whereas umbilical cord androgens levels are similar to the normal pregnancies in some studies [120], higher levels of testosterone have been shown in others [19, 121]. A novel method that measures the sebum excretion on newborns have demonstrated that newborns born to mothers with PCOS excrete more sebum at birth compared to those born to control mothers [122]. Maternal androgen exposure increases 5α reductase (SRD5A) activity, but not the activity of 11β-hydroxysteroid dehydrogenase in 3 years old daughters of women with PCOS, suggesting a hyperandrogenic state that could contribute to the development of PCOS in the extra-uterine life [123]. Moreover, daughters of women with PCOS from Chile display hyperinsulinemia and augmented ovarian volume before puberty that persist during pubertal development, which placed them at higher risk for metabolic and reproductive derangements [22]. Sons born to PCOS mother are also affected and exhibit higher body weight from early infancy and insulin resistance when the subjects got older, placing them at risk for the development of T2D and cardiovascular disease later in life [23].

Obesity is associated with preterm deliveries and increased rates of infant mortality [124]. Moreover, children of obese mothers have an increased incidence of attention deficit hyperactivity disorder (ADHD), anxiety, and other psychiatric disorders at 3-5 years old [125]. Also, a strong association with cerebral palsy in newborns born to obese mothers at term has been found [126]. Later in the life, offspring from mothers who were obese during pregnancy have higher risk of hospital admission for cardiovascular events [127] and significant associations between increased maternal BMI, weight gain during pregnancy, and greater offspring waist circumference, BMI, and fat mass index at 30 years old is reported [128]. Moreover, sons of obese mothers have higher risk to display cryptorchidism and hypospadias [129].

Although the majority of the human studies state association and not causality, animal models of maternal androgen excess have also demonstrated the deleterious effects in offspring, mostly through intergenerational studies approach. Indeed, maternal androgen excess exposure is a well-established animal model proved in rodents, sheep, and non-human primates. In those models, the offspring are usually affected by intrauterine growth restriction, whereas in adult

age, they develop a PCOS-like phenotype including reproductive, metabolic and behavioral alterations [130-135].

Studies evaluating the effects of maternal obesity have demonstrated altered placental function [106], fetal overgrowth [102], hypertension, glucose intolerance [101], stress [104] and even altered methylation profile in oocytes and liver from offspring, predisposing them to develop metabolic failures [105]. Obesity also alters metabolic, vascular and anatomical impairments in the extra-uterine life [136, 137] such as leptin resistance and hepatic steatosis, hypertension, dyslipidemia and obesity [137].

Further, female mice born to mothers fed high fat high sugar diet display impaired insulin sensitivity related to mitochondrial failure in skeletal muscle, that was transferred to the next two generations (up to F3) even when F1, F2 and F3 generations were fed with "healthy diet" [138]. The propagation of the mitochondrial failure suggest an increased risk to develop metabolic disturbances in the future generations [138].

1.5.1 Placenta dysfunction in PCOS and obesity

In PCOS, the adverse pregnancy state, fetal outcomes and long term consequences could be, at least in part, explained by alteration in the placental steroidogenic process. In the placenta from women with PCOS a higher activity of 3β-hydroxysteroid dehydrogenase type 1, which catalyzes the conversion of DHEA to A4, and a decreased activity of CYP19A1 which converts A4 to estrone and 16–hydroxytestosterone to estriol, and testosterone to estradiol [139-141] (Fig.4) is reported. These changes suggest an augmented capacity to maintain an androgenic state [119].

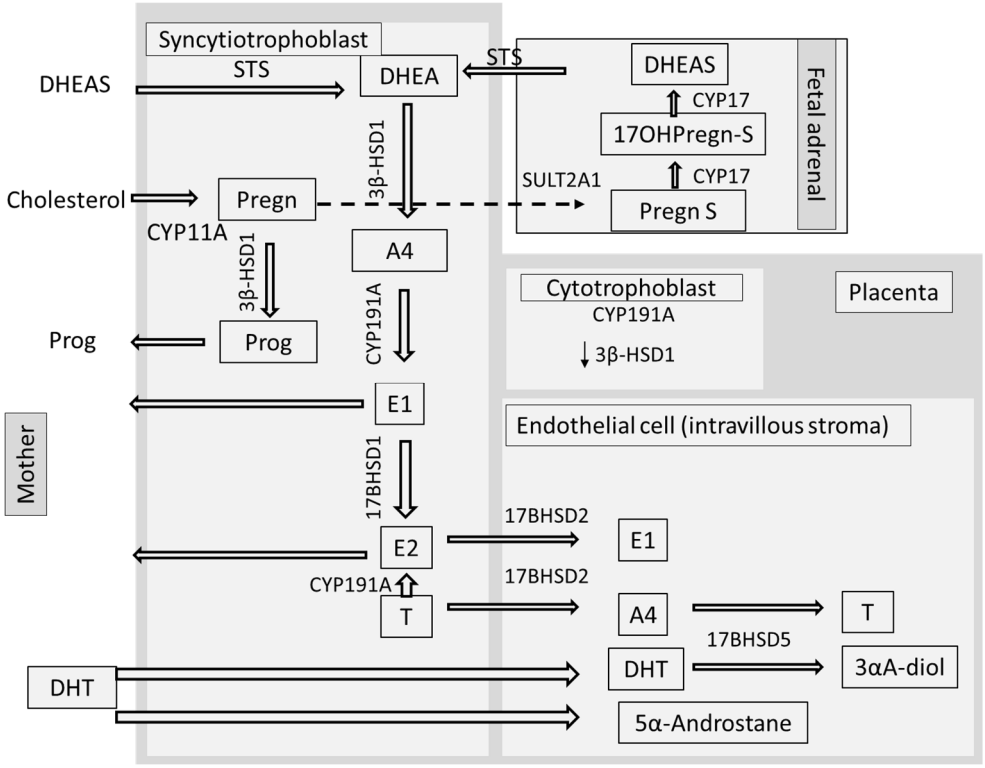


Figure 4. *Simplified view of steroidogenesis pathways in human placenta. Placenta is not capable to synthetize androgens de novo because it lacks CYP17 enzyme. Then the placenta utilize the maternal and fetal DHEAS to produce androgens (A4 and T) that subsequently are converted into estrogens by CYP19A1.*

Placenta nutrient transport is dependent on placenta size, morphology, nutrient transporter capacity and availability, utero and feto-placenta irrigation [142] and to genetically expected and the environmental interferences [143]. For the nutrition of the fetus, the nutrients have to cross the placenta trough two selective membranes: the microvillus membrane (MVM) that faces the maternal side and the basal membrane (BM) that faces to the fetal side and is closer to fetal capillaries [144]. Whereas glucose is transported by facilitated diffusion, amino acids are transported by proteins included in system A and L. As triglycerides cannot cross the sinciciotrophoblast they should first break down into FFA by placental triglycerides lipases: lipoprotein lipases and endothelial lipases [145, 146]. Later the FFA are transported by fatty acid transport proteins (FATP), fatty acid translocase (FAT/CD36), plasma membrane fatty acid binding protein (FABPpm), and fatty acid binding proteins (FABP) [146, 147].

In pregnant rats, a dose of 0.5 mg/kg of testosterone propionate in late pregnancy decreased fetal body and placenta weight [148] in both male and females [149]. In the same model, the authors reported that, even the fetal levels of testosterone were not affected [149], maternal androgen exposure caused a down-regulation of placental amino acid transporter expression and a decrease in placental amino acid transfer, which in turn decreased the fetal and placental

weight. Therefore, the authors suggest that maternal androgen exposure would be able to affect the fetal development mediated by changes in placental function [149].

Mammalian target of rapamycin (mTOR), and signal transducer and activator of transcription 3 (STAT-3) have been proposed as the major regulators of nutrients transport in placenta [150]. In animal models of fetal growth restriction, the lower protein intake inhibits placental insulin, mTOR and STAT-3 signaling that is associated with a down-regulation of placental amino acid transporters [151]. On the other hand, the high calorie intake increases the expression, through mTOR signaling activation, of some amino acid transporters and increase the activity of the system A and L in placenta, leading to increased fetal growth [152].

1.6 TREATMENT OF PCOS DURING PREGNANCY

1.6.1 Lifestyle modifications

There is scarce scientific literature about weight management in pregnant women with PCOS despite of being considered as the first-line treatment strategy in PCOS recommended by evidence-based guidelines. Weight management gain is defined as prevention of excess weight gain, weight loss, or maintenance of a reduced weight, through lifestyle behavioural interventions that include diet and physical activity [153].

1.6.2 Pharmaceutical treatments

Due to the heterogeneity of the syndrome where multiple organ systems are involved in its development, it is difficult to establish specific treatments. One of the pharmaceutical approaches used is the insulin sensitizer metformin. Its action has been related with improved uterine artery blood flow as demonstrated by reducing the uterine artery impedance from first to second trimester in women with PCOS [154], but the reduction of complications is less clear in this sense [154, 155]. Vanky et al demonstrated that despite not reducing circulating androgen levels during pregnancy, none of the women receiving metformin had severe pregnancy complications (0/18). In the placebo group, 7/22 women experienced at least one severe complication either during pregnancy or during partum [156]. Metformin also decrease the rate of pregnancy loss and preterm delivery [157]. Moreover, metformin decreased the amount of cell free fetal DNA, an indicator of pregnancy complications, in plasma of PCOS women compared to women that followed 12 weeks exercise management during pregnancy [158]. Surprisingly, metformin has shown no effects on glucose homeostasis in pregnant women with PCOS [159]. Glueck et al show that metformin in combination with dietary counselling prior to pregnancy is able to reduce: insulin secretion and testosterone, insulin

resistance, preconception weight, and maintaining these effects throughout pregnancy [160]. However, those results are questionable because the study did not include a placebo group.

1.6.3 Electroacupuncture (EA)

Acupuncture is an ancient therapy used by oriental cultures since more than 3000 years. The technique comprises the insertion of thin sterile needles (usually stainless steel) in the body with a variable depth. After insertion, the needles are stimulated manually or by electrical stimulation with high (100Hz) or low frequency (1-15 Hz), which is called electroacupuncture (EA). Western medical acupuncture is an adaptation of the Chinese approach and use current knowledge of anatomy, physiology, pathophysiology and evidence-base medicine [161].

The insertion of needles in muscles activates somatic afferent nerves fibers, predominantly thick myelinated Aβ, thin myelinated Aδ and thinner unmyelinated C fibers [162]. This stimulation has simultaneous local (peripheral), segmental (spinal cord) and central (supraspinal) effects (Fig. 5) [163, 164]. The stimulation causes a peripheral release of neuropeptides that include substance P (SP), calcitonin gene-related peptide (CGRP), vasoinstestinal peptide (VIP) and nerve growth factor (NGF) [165]. At spinal area, acupuncture stimulate sympathetic reflexes of organs located in the same innervation area as needle placement [164, 166]. Simultaneously, the transmission of stimulation continues to the central nervous system (CNS) modulating hypothalamus and pituitary function [163, 167-170]. Of note, the muscle contraction provoked by EA results in similar effects that exercise does. In fact, both EA and exercise modulate the release of endogenous opioids.

Acupuncture has been shown to influence visceral blood flow, an effect that seems to be mediated by modulation of somato-autonomic reflexes [171, 172]. Furthermore, acupuncture with low-frequency electrical stimulation of the needles has been demonstrated to increase ovarian blood flow response trough the modulation of sympathetic nerve fibers innervating the ovary [166, 169, 173].

Studies have shown that in non-pregnant women undergoing in vitro fertilization, eight acupuncture treatments with low-frequency EA increase uterine artery blood flow [174], with similar effects in non-pregnant rats [166, 173]. Zeisler et al evaluated the effects of acupuncture on uterine artery blood flow during healthy pregnancies [175]. In that study, acupuncture decreased the systolic/diastolic ratio in umbilical artery, without affecting the fetal heart rate after a single bout of acupuncture. However the study was conducted in normal pregnancies near term when umbilical blood flow has reported to be maximal [175].

There is a lack of reports about the use of acupuncture in pregnancies with complications, for example preeclampsia or gestational diabetes. During pregnancy, the effect of acupuncture for pelvic girdle pain has been found superior to exercise and self-management group [176, 177], although no better when compared to sham acupuncture [178]. Low-frequency EA has been shown to decrease the need of epidural anesthesia although without a significant decrease in pain [179], and to decrease the length of the active phase of labor [180].

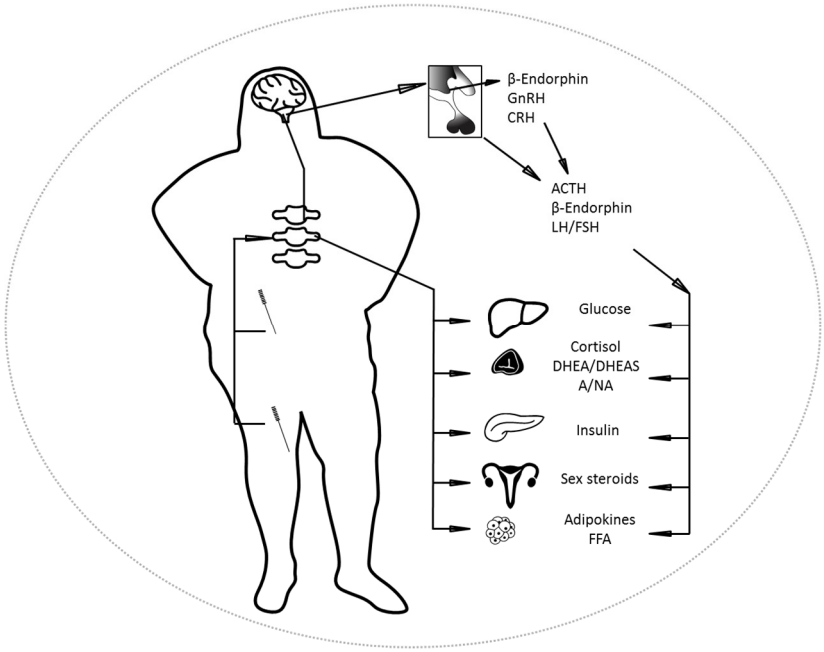


Figure 5. *Theoretical model of the effect of low-frequency electroacupuncture (EA) in PCOS. Electrical stimulation of needles inserted in skeletal muscles activate ergoreceptors that in turn activate afferent nerve fibers Aβ, Aδ and C fibers. That activation has a: 1) segmental effect, where the autonomic spinal reflexes can modulate the functioning of organs in the same area of innervation of the fibers stimulated by EA; 2) central nervous system effect, activated by ascending pathways that stimulate different areas in the brain, as hypothalamus and pituitary and therefore modulating nervous and endocrine system. Indeed, the release of β-endorphin by acupuncture mediate, in turn, the secretion of GnRH and CRH by the hypothalamus with the subsequent modulation of hormones from pituitary (LH/FSH, ACTH) and finally modulating the reproductive, adrenal hepatic and pancreatic function. (ACTH: adrenocorticotropin hormone; GnRH: Gonadotropin releasing hormone; CRH; Corticotropin hormone; FSH: follicle stimulating hormone; LH; Luteinizing hormone; NA: noradrenaline; A: Adrenaline)*

2 AIMS

2.1 GENERAL AIM

The overall aim of this thesis was to determine the role of androgens and obesity during pregnancy and whether acupuncture could modulate placenta dysfunction and fetal growth.

2.2 SPECIFIC AIMS

Paper I

To determine whether PCOS in women without pregnancy complications affect placental signal transducer and activator of transcription 3 (STAT3) and mechanistic target of rapamycin (mTOR) signaling.

Paper II

To test the hypotheses that maternal androgen excess decreases placental and fetal growth, and modulates placental expression of markers of steroidogenesis, angiogenesis and sympathetic activity, and that low-frequency EA prevents these changes.

Paper III

To evaluate maternal growth and metabolism, placenta weight, placenta steroid receptor expression, and fetal growth in mice exposed to maternal androgen excess with or without maternal obesity.

Paper IV

To explore how maternal androgen exposure with or without HF/HS-induced obesity affect the total and phosphorylated proteome of placenta and fetal liver, with the aim to find novel target and pathways that could explain altered placenta function and the development of fetal organs and their functions.

3 METHODOLOGICAL CONSIDERATIONS

3.1 ETHICS

All the studies presented here are in accordance to the Declaration of Helsinski (Paper I and IV) or according with the legal requirements of the European Community (Decree 86/609/EEC) (Paper II, III and IV). Moreover, the studies were approved by the Research Specific Ethics at Uppsala University, Sweden (Dnr 2011-372) and Midt-Norge (145-04), (paper I); Animal Ethics Committee of the University of Gothenburg (Dnr: 53-2013, paper II), and (Dnr: 116–2014, paper III), and Animal Ethics Committee of Karolinska Institute (259-14, with addendum N263-15, paper IV).

3.2 DESIGN

3.2.1 Clinical study (paper I)

Placenta tissues and blood were obtained from:

- The PregMet Study, conducted at St.Olav´s Hospital, University Hospital of Trondheim, Norway.
- Basic Biobank at Uppsala University, Sweden.

Inclusion criteria:

- PCOS: Diagnosis of PCOS according to Rotterdam criteria [24] by a gynecologist based in the documentation before pregnancy.

Exclusion Criteria:

- Preeclampsia
- Gestational diabetes
- Chronic disease as hypertension, kidney disease and diabetes
- Multiple gestation
- Other than scandinavian heritage

Samples:

- 40 Control pregnant women were selected from BASIC Biobank and 14 women with PCOS are from BASIC and 24 from PregMet study (Fig. 6)
- In all women a venous blood sample was obtained at delivery. After clotting, serum was separeted by centrifugation and frozen at -70̊C.

- After vaginal delivery or cesarian section, placentas were washed in sterile phosphate-buffered saline and snap frozen at -70̊C.

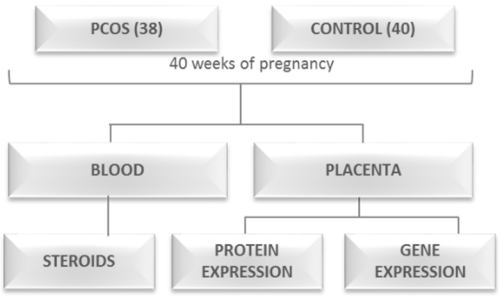


Figure 6. *Design of study paper I. Blood and placenta were collected from pregnant women with PCOS and control at term. Circulating steroids were analyzed in serum samples. Protein and gene expression of molecules involved in steroid action, metabolic pathway and cytokines were assessed in placenta.*

In paper IV, due to the identification of a novel phosphorylation of COMT in placenta and fetal liver, we aimed to study whether the same or a similar phosphorylation was present in human placenta. Therefore, placental tissues from normal weight women with (n=5) and without PCOS (n=4), and from overweight/obese women with (n=5) and without PCOS (n=5) were selected from BASIC Biobank to analyze targeted proteomic analysis of the COMT enzyme (Fig.7C, bottom part). Of note, women with female fetus was included in this analysis.

3.2.2 Animal studies (paper II-IV)

The design of the experimental studies included in paper II-IV are shown in Figure 7. Overall the studies aimed to investigate the effect of maternal hyperandrogenemia in the late pregnancy on fetal and placental development.

In paper II, time pregnant Wistar (Charles River, Germany) rats were received at gestational day (GD) 7 (Fig.7A). Animals were fed normal chow and were injected with testosterone propionate (TP) (0.5mg/Kg) or vehicle from GD15 to 19. From GD16 to 20 the animals were treated with low frequency EA or handling. The sacrifice was done during GD 21 after 4 hours of fasting. In this study the dose of TP was selected in order to model the two-fold increase in circulating testosterone previously reported [149, 181].

In paper III and IV a combination of the prenatal androgenization (PNA) mouse model with a high-fat/high-sugar (HF/HS) diet-induced obese mouse model that has been shown to deliver

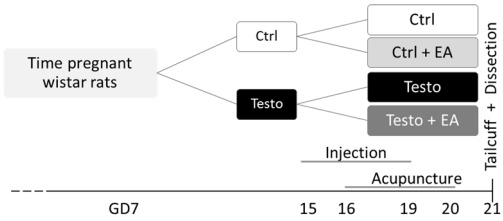
LGA offspring was used [102]. The phenotypic characteristics were evaluated by studying maternal growth and metabolism, placenta weight, placenta steroid receptor expression, and fetal growth (paper III, Fig.7B). A proteomic analysis of placenta and fetal liver in mice exposed to maternal androgen excess with or without obesity was performed (paper IV, Fig.7C). In studies III and IV mice were used, instead of rats, because of the opportunity to work with either transgenic or knock–out animals in the future, and because of the availability of antibodies and assays available for mice. Injection of DHT (250µg by body weight calculated from a 20g standard mouse) in a mixture of benzyl benzoate and sesame oil between GD 15.5 and 17.5 (paper III) was used to induce prenatal androgenization. In paper IV a fixed dose of 250µg was injected between GD16.5 and 18.5 in order to follow the original model described by previous authors [132, 182-185].

3.2.3 HF/HS obesity (papers III and IV)

The obese mouse model used in this thesis aimed to induce the phenotype previously reported by Rosario *et al* with increased fat mass, glucose intolerance, high maternal circulating insulin, leptin, and cholesterol and a decrease in circulating adiponectin and overgrown fetus [102, 152], and to combine it with the maternal androgen exposure model. In brief animals were fed:

- High fat/high sucrose diet (HF/HS): 40 Kcal% fat, 43% carbohydrate and 17% of calories from protein, plus 20% of sucrose in water supplemented with vitamins and minerals for 4 to 10 weeks until the animals in HF/HS group reached 25% increase in BW (paper III, Fig. 7B) or for 10 weeks (paper IV, Fig.7C).
- Control diet (CD) 10 Kcal% fat, 73% carbohydrate and 17% of calories from protein.

A



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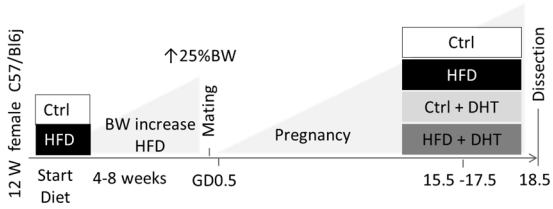

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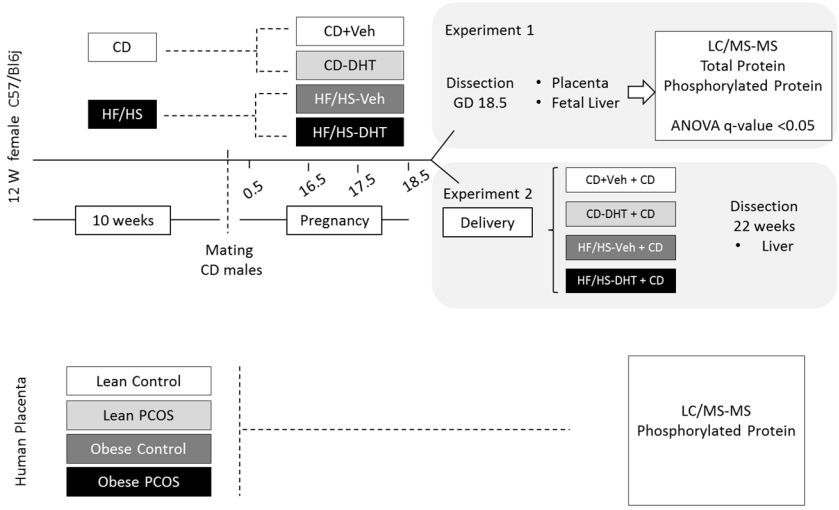

Figure 7*. Design of studies in papers (A) II, (B) III and (C) IV.*

3.2.4 Treatment with low frequency electroacupuncture (paper II)

The treatment with low-frequency EA was started on GD16, one day after the androgenization began. The procedure was done in conscious rats. In order to control for environmental factors, all rats, injected with either vehicle or testosterone received the same handling. Rats were wrapped up in a special homemade fabric harness that allows to insert the needles in the abdominal area and in the hind limbs. In the EA group, acupuncture needles, 0.20 mm in diameter and 15 mm in length were used. Two needles were inserted into rectus abdominis and two needles were placed into the triceps surae muscle in each hind limb. To provoke muscle contractions, all needles were connected to electrodes attached to an electrical stimulator and stimulated with 2 Hz frequency burst pulses. The length of the treatment on the first day was 15 minutes and it was increased to 20 minutes the following days. As all the needles could potentially trigger a physiological response, in the control group rats were only handled and no needles were inserted.

3.2.5 Summary of the methods across the papers

Table 1. Summary of methods used across the studies

	Human Study	Experimental Studies		
Method	Paper I	Paper II	Paper III	Paper IV
Breeding			•	•
Body composition (DEXA)			•	
Tail-cuff		•		•
OGTT			•	
Assay				
Western Blot	•	•	•	
qPCR	•		•	•
ELISA		•	•	•
Mass spectrometry				•
HPLC or GC-MS/MS	•		•	•

3.3 ANIMAL ASSESSMENTS

3.3.1 Dual Energy X-ray Absorptiometry (DEXA)

With DEXA, the body composition analysis allows to investigate not only bone mineral content (BMC), but also fat mass (FM) and lean body mass (LBM) [186]. DEXA has the advantages of low cost, low radiation, short evaluation times and conserve the animal for more studies in the same protocol. Disadvantages are that DEXA measure all body lipid content and not only the adipose tissue and secondly, animals have to be anesthetized.

This method was used in paper III to evaluate the body composition the day before mating and at GD18.5 to investigate how body composition changed during pregnancy.

3.3.2 Body composition analyzer (BCA)

Nuclear magnetic resonance is a non-invasive, in vivo body composition analysis used in small animals, to image and quantify subcutaneous adipose tissue and lean mass. The method used in this thesis is time domain nuclear magnetic resonance (TD-NMR, Bruker) and was used to evaluate body composition of fetus in paper III.

3.3.3. Oral glucose tolerance test and Insulin sensitivity index (HOMA-IR)

The OGTT measures the ability of the body to clear the glucose from blood circulation and was used in paper III. Oral gavage include the incretin effect and is to be preferred above intraperitoneal injection [187]. In brief, after 6 hours of fasting, glucose measurements were

taken at basal level (time 0) and at 15, 30, 60 and 90 minutes after 50 mg of glucose/250µl NaCl 0.9% (20%, p/v). Blood samples for insulin measurement was taken at 0 and 15 minutes was taken. Although OGTT does not distinguish between peripheral insulin resistance and β-cell dysfunction, different index could be calculated upon the fasting glucose and insulin levels to evaluate insulin sensitivity, as the HOMA-IR, or β-cell dysfunction with HOMA-β.

3.3.4 Tail-cuff

Tail–cuff plethysmography or photoplethysmography is a non-invasive method that allows measuring arterial pressure, specifically systolic blood pressure. In brief, a light illuminates a spot in the tail of the animal and pressure oscillations in the cuff related to changes in blood flow during release of the occlusion are sensed and measured by an aneroid manometer. For better measurements, the animal should be anesthetized, although is important to note that anaesthesia could decrease blood pressure. This method was used in paper III were the arterial pressure was evaluated in pregnant rats at GD21. In paper IV, CODA System was used as a non-invasive system for measuring arterial blood pressure. It is a volumetric based method to measure the blood flow and blood volume in the tail, where no artefacts related to ambient light are present. For the measurement, a special animal holder maintains anaesthesia in mice to avoid stress while the measures are taken.

3.4 PROTEIN EXPRESSION ASSAYS

3.4.1 Western Blot

This is a widely used technique aimed to imunodetect proteins that have been separated by native or SDS-polyacrylamide gel electrophoresis and transferred onto a nitrocellulose or polyvinylidene difluoride (PDVF) membrane, most commonly trough electrophoretic transfer (electroblotting). Once the proteins are transferred, the membrane is incubated with specific antibodies followed by a subsequent visualization of the labeled protein, usually by chemiluminiscence. As a ladder is loaded in the same membrane, the identification of the target proteins is based on the visualization of the bands that should be at a specific size indicated the ladder. Depending of the antibody used, the protein could be detected in its unphosphorylated or phosphorylated form. In this thesis at least three systems that involve different technologies in electrophoresis, blotting or detection were used, but with the same principles listed above. Paper I used Invitrogen technology. In paper II, two methods were used, because method was

used in different universities: i) Bio-rad system (also used in paper III), and ii) the classical method (homemade gels and detection and quantification using x-ray films).

3.4.2 Enzyme-linked immunosorbent assay (ELISA)

ELISA is a plate-based antibody assay technique designed for detecting and quantifying, proteins, hormones, among others. The detection antibody can be covalently linked to an enzyme, or can itself be detected by a secondary antibody that is linked to an enzyme through bioconjugation.

3.4.3 Mass spectrometry

Gas, liquid or ultra-high performance liquid chromatography/ mass spectrometry (GC/MS-MS or LC/MS-MS, UPLC- MS-MS, respectively) are analytical techniques that by physical separation, ionization and ions sorting, detect different components of a sample based on their mass-to-charge ratio. In this thesis, circulating steroids (paper I, II) were measured by either GC/MS-MS (paper I) or UPLC- MS-MS (paper III). Global proteomic analysis was done by LC/MS-MS (paper IV).

3.4.4 Polymerase chain reaction and Real-time PCR (qPCR)

PCR is based on using the ability of the enzyme DNA polymerase to amplify a DNA template to produce specific DNA fragments (amplicons) *in vitro*, by assembling free nucleotides using single-stranded DNA as a template and DNA oligonucleotides or primers to initiate DNA synthesis. PCR is a common method for amplifying DNA, and for measuring mRNA, where the mRNA sample is first reverse-transcribed to complementary DNA (cDNA). The use of primers or fluorescent DNA-binding dyes to detect and quantitate a PCR product allow quantitative PCR to be performed in real time (Real time PCR), where DNA or cDNA copy number can be established after each cycle of amplification. It is carried out in a thermal cycler that has the capacity to illuminate each sample with a beam of light at one specified wavelength and detect the fluorescence emitted by the excited fluorophore. Finally, quantitative PCR data can be expressed relative to an internal standard (relative quantification) used in this thesis, or relative to a standard curve.

3.5 STATISTICS

Statistical Package for Social Sciences (SPSS) and graphpad were used for statistical analyses. In paper IV, was R studio program used to perform two-way ANOVA. Most of the data are

shown as mean ± standard error of mean (SEM) and a p value <0.05 was considered as significant.

In Paper I statistical differences were calculated with Fisher permutation test and categorical data were calculated by using χ^2 test. Correlation were calculated by Spearman's test.

Paper II, Kruskal Wallis test followed by Mann Whitney *U* test for group comparison were used. Non parametrical test was used because in most comparison the groups had less than 10 samples.

With the objective to analyze the effect of diet or injection factors, a two way ANOVA followed by Bonferroni correction was selected to compare those factors (Paper III and IV). In proteomic analysis (paper IV), all data were log2-transformed and missing values were imputed from normal distribution. All data were filtered by one-way ANOVA tests with Benjamini-Hochberg correction for multiple testing. False discovery rate (FDR) correction with a *q* value of 0.05 and at least 25 % in fold change in the subsequent t-test between the groups was used to filter the data of interest in each data set. After this filtering, selected proteins were analyzed by two-way ANOVA in order to investigate if it was a main effect of diet or injection factors, or an interaction of the main effects in the differential expression of proteins.

4 SUMMARY OF RESULTS

4.1 PAPER I

Abnormal steroidogenesis and dysregulated placental metabolic pathways in pregnant women with PCOS

Women with PCOS have an altered steroidogenesis as demonstrated by elevated circulating androgen levels and decreased estradiol. Moreover, alterations in protein and gene expression of molecules related to energy homeostasis and steroidogenesis were found in placenta from women with PCOS. A higher phosphorylation of *STAT-3* together with a decrease of *SCL2A4* and *CYP11A1* mRNA and an increase of *17β HSD* (*ARKI C3*) mRNA were found. Although *STAT-3* was activated, the downstream effectors 4EBP1 and S6 ribosomal protein remained unaffected in placenta.

4.2 PAPER II

Electroacupuncture impair fetal and placental development in rats exposed prenatally to testosterone.

EA in pregnant rats injected with testosterone increased systolic blood pressure and decreased circulating norepinephrine and corticosterone. In rats exposed to maternal androgens, contrary to our hypothesis, the fetal and placental weight was decreased and there was an impairment in the angiogenic pathways as demonstrated by a decrease in protein expression of placenta VEGFR1 and augmented VEGFA/VEGFR1 ratio. Moreover, a decreased protein expression of proNGF protein expression, together with increased mature NGF (mNGF) and altered mNGF/proNGF ratio was also present. Of note, EA in control rats did not affect any of the variables studied.

4.3 PAPER III

Diet induced maternal obesity differentially affect maternal and fetal triglyceride metabolism in liver

Mice fed HF/HS diet had higher fat content before mating and at GD18.5 with no difference in glucose homeostasis, whereas HOMA-IR was decreased in dams exposed to DHT during pregnancy. At GD18.5, the placental androgen receptor (AR) protein expression was increased compared with controls. Maternal livers weighed more in mice fed with HF/HS regardless of DHT injection and the triglyceride content was higher in their livers with a higher mRNA expression of *Fitm1* and *Pparg*. Diet, injection or the interaction of both factors dysregulate the mRNA expression of enzymes related with *de novo* lipogenesis. In fetal livers from dams fed HF/HS-diet, the triglyceride content was lower

as well as mRNA expression of *Srebf1c*. Prenatal DHT exposure decreased *Pparg* mRNA expression in fetal livers.

4.4 PAPER IV

Diet induced maternal obesity and androgen exposure affect the catecholamine metabolism in placenta and fetal liver

Circulating noradrenaline was decreased in animals injected with DHT. In placenta, the phosphorylation of ATP-citrate synthase ($ACLY^{547}$) was decreased in mice fed HF/FS diet and an interaction between maternal diet and DHT exposure dysregulated the phosphorylation of Catechol-O-Methyltransferase ($COMT^{S261}$). In female fetal livers, the phosphorylated $COMT^{S261}$ was increased due to maternal obesity. Moreover, in liver from female offspring at adult age the gene expression of COMT was affected by the interaction of both factors, diet and DHT exposure.

5 DISCUSSION

Many studies in the field of PCOS investigate pathophysiology of infertility and its long-term metabolic and behavioral health consequences, but less are focused on the effects of PCOS in pregnancy and its potential consequences on offspring health and ultimately the developmental origin of the syndrome.

Pregnant women with PCOS are at higher risk to have pregnancy complications with health consequences in the offspring. In fact, women with PCOS are at higher risk to have preeclampsia or pregnancy induced hypertension [188, 189], gestational diabetes mellitus [15] and preterm deliveries [17, 190]. Babies born to mothers with PCOS are at higher risk to be born LGA [15, 18, 189] or SGA [15, 189, 191]. Of note, the SGA babies are smaller than the SGA in the control group [191]. Women fulfilling all three PCOS criteria before pregnancy are the more affected with pregnancy complications [86]. It is interesting to note that it is the risk of developing preeclampsia and gestational diabetes that is increased among women with PCOS, and no other complications, suggesting that there are specific pathophysiology mechanisms. It is not clear whether the hyperandrogenemia is the key factor in the development of these complications.

In order to avoid any confounding effect of pregnancy complications, in the first article we only included women with and without PCOS with uncomplicated pregnancies. Pregnant women with PCOS showed higher levels of androgens as previously reported [14, 85].

We found a higher phosphorylation in STAT-$3^{Tyr\text{-}705}$. STAT-3 is recognized to regulate placental nutrient transport [151], angiogenesis [192] and fetal growth [151]. Interestingly, STAT-3 has been found to be induced by glucose *in vitro* [193] and also be highly phosphorylated in placentas from preeclamptic patients [194], although lower phosphorylation in severe preeclampsia [195] has also been found. It is known that leptin activates JAK-STAT signaling pathway, which in turn regulates system A amino acid transport activity [150]. However, mRNA of leptin and the leptin receptor was decreased in the placenta from women with PCOS, whereas phosphorylated STAT-3 was upregulated [196]. Interestingly, the activation of STAT-3 could be due to an inflammatory state [197] as was also suggested in paper II, where placentas from rats prenatally exposed to testosterone expressed higher level of p-JNK. JNK is a member of mitogen-activated protein kinase (MAPK) family that is related to inflammation [198], induced by cytokines but also by testosterone *in vitro* [199].

Contrary to our hypothesis, dams injected with testosterone and treated with EA had increased systolic blood pressure, and a decrease in circulating norepinephrine. Many articles have

published beneficial effects of EA in lowering the blood pressure [200-202]. We hypothesized that EA, via somatic afferents, could modulate the sympathetic activity and increase blood flow as demonstrated in humans [174]. During EA treatment, sympathetic activity has been shown to increase [203, 204], followed by a decrease post treatment [205, 206]. In rats exposed to testosterone and treated with EA, we found decreased fetal weight in female fetuses and placenta. That could be a consequence of a decrease in the angiogenesis process suggested by the lower protein expression of VEGFR1 and a high VEGFA/VEGFR1 ratio in the same group of animals. Interestingly, patients with PCOS have elevated blood pressure during pregnancy [90] and patients with preeclampsia have increased circulating free testosterone [207] and decreased placental aromatase expression [208]. Moreover, higher protein expression of androgen receptor and lower protein expression of aromatase in placenta from patients with gestational diabetes have been shown [209]. All these findings, reinforce the idea that there is a link between higher androgens and gestational complications in PCOS that could be mediated by common molecular pathways.

As the effect of EA is in part mediated via the sympathetic nervous system, we measured proNGF and NGF as markers of sympathetic activity [210]. In animals exposed to testosterone and treated with EA, an increase of mNGF and mNGF/proNGF ratio in placenta was found. In human placenta the NGF is localized in cyto- and syncytiotrophoblast, as well as in the extravillous trophoblast and decidual cells [211] and almost no stain has been shown in the fetal part [211]. NGF controls folliculogenesis and also angiogenesis [212, 213]. It is known that under stress, upregulation of NGF in decidua would induce a pro-inflammatory environment. The results of that condition is a pro-abortive environment, mediated by the stimulation of the expression of adhesion molecules intercellular adhesion molecule 1 (ICAM1) and selectin platelet (SELP) and their ligands. Further, the neutralization of NGF in the maternal interface increase the infiltration of $TRKA^{+}$-NK cells to the decidua, also deriving in local inflammation and abortion [214]. Of note, the VEGFR1 blockage have shown to have an effect in reducing inflammation but also angiogenesis [215]. Therefore, low-frequency EA in the animals injected with testosterone and treated with EA would have been exposed to an increased inflammatory environment given by the imbalance of NGF that the VEGFR1 downregulation would not be able to normalize and affecting the angiogenesis process that is associated with a lower fetal and placental weight.

As in PCOS, obese women by themselves also display infertility [216, 217], miscarriages [218], adverse pregnancy outcomes as preeclampsia [219, 220] and gestational diabetes [221, 222]. It is known that the combination of obesity with PCOS is considered to be more deleterious [18, 86, 189, 190, 223]. In paper III and IV, we aimed to combine the well

characterized PNA mouse model [132, 182, 184, 185] with a maternal obesity model [102, 152]. Contrary to what expected, the weight of the fetus was not different to the control group. One reasonable explanation might be the lower weight gain in dams fed HF/HS compared to dams on control diet. Alternatively, the diet may have cause a mild phenotype, although this was not demonstrated in the circulating adiponectin or glucose metabolism, as indicated by unaltered HOMA-IR. Surprisingly HOMA-IR was decreased in dams exposed to DHT indicating an acute effect of DHT. The lower increase in body weight during late pregnancy is in line with other studies in humans [224, 225] and animal models, using a similar HF/HS-induced obesity model with the same litter size [226]. Our results are supported by a previous *in vitro* study of glucose-induced insulin secretion in cultured beta cells from normal rats [227]. Exposure for 72 hours to either DHT or testosterone reduced the insulin secretion, and the effect was reversed by the antiandrogen flutamide, demonstrating the capacity of androgens to modulate insulin secretion via androgen receptors [227].

Because maternal androgens have the potential to affect the fetus and/or placental function [149], it is important to investigate the circulating levels of androgens and other circulating sex steroids. No previous studies using the maternal androgen excess model have measured DHT in maternal serum in mice [132, 182, 184, 185]. Progesterone was higher in the HF/HS group than in control animals regardless of DHT exposure. That is in line with a recent report that shows an increase of progesterone and a reduction of IFN-γ expression in NK cells that is known to be involved in supporting uterine spiral artery remodeling [228].

Liver weight and triglyceride content increased in dams fed HF/HS with a tendency to be higher in the obese dams exposed to DHT. It is known that obesity, specifically with large amount of visceral adipose tissue contributes to a high prevalence of non-alcoholic fatty liver disease (NAFLD) [229]. Moreover, the prevalence of nonalcoholic fatty liver disease in women with PCOS has been shown to be 25% [230] and hyperandrogenic women with PCOS exhibit higher liver fat content than women with PCOS without hyperandrogenism, independently of BMI and insulin resistance [231]. In this study, the high triglyceride amount in liver from obese dams with or without hyperandrogenism could respond to different stimulus or have synergic effect in the case of obese dams injected with DHT. Moreover, all non-invasive indices of hepatic steatosis and hepatic fibrosis are higher than normal in women with PCOS, and even highest when the metabolic syndrome is present [232].

The molecular signatures behind the triglyceride accumulation were different across groups, but most of the changes were due to HF/HS induced obesity factor. In summary, lipid accumulation in maternal liver does not seem to be related to *de novo* lipogenesis demonstrated

by the decrease rather that increase in the mRNA expression of genes that constitutes the pathway [233]. Obese dams showed increased expression of *Pparg* in the liver, which is known to be related to HF/HS-induced steatosis [234, 235] although its participation is controversial, because its activation by thiazolidinedione's reduces hepatic steatosis [236, 237]. It is known that *Pparg* overexpression results in increased expression of fat storage-inducing transmembrane protein *Fitm1* [238], a precursor of lipid droplet formation [239]. Therefore, it is possible that the increased expression of *Fitm1* that we observed in the livers of dams fed with HF/HS constitutes a link between increased expression of *Pparg* and accumulation of TG in the liver of obese dams that was not modulated by DHT.

The liver TG content was, on the contrary, decreased in fetus from dams fed with HF/HS and no changes in mRNA expression of genes related to *de novo* lipogenesis was found. Which is in contrasts to the higher lipid droplet accumulation in fetal livers from dams fed HF/HS previously shown [240]. Similar to our results, Abbott *et al.* showed a lower concentration of circulating FFA in female rhesus monkey fetuses from mothers that were androgenized, and who increased their body weight during pregnancy. [241] Of note, the FFA levels increase in adult female offspring [242]. One possible cause of the lower accumulation of TG in the fetal liver from obese dams could be a consequence of modifications of placental transfer of FFA [240], that is believed to be an important source for TG synthesis in the fetal liver [147]. Another possibility is that β-oxidation is activated in the fetal liver in obese dams, which was not the focus of this study. In those fetuses, the expression of *Srebf1c* was decreased which could be related to lower TG content [243, 244]. Interestingly, the expression of the transcription factor *Pparg* was decreased in livers from fetuses exposed to DHT, which is most likely related to a direct action of androgens on fetal liver. Of note, the effect of androgens on *PPARg* has been shown before where testosterone and DHT inhibit adipocyte differentiation *in vitro* by decreasing the gene expression of *PPARg*, mediated by the impairment of Bone Morphogenic Protein 4 (BMP4) signaling [245]. Moreover, we have demonstrated that in adipose tissue of women with PCOS the expression of *Pparg* mRNA is decreased compared to controls [246]. As it is not clear whether androgens could cross the placenta [20, 119], the higher amount of circulating androgens in maternal circulation could provoke changes in the placenta that could be harmful to the fetus, as in the case of the modulation of the expression of mRNA of *Pparg*. The placental protein expression of androgen receptor in DHT exposed dams was higher independent of diet, similar to what we have previously reported in the placenta from testosterone-exposed pregnant rats [247]. Of note, at adult age, female offspring displayed higher levels of oil red O staining in livers and high circulating testosterone and TG in the absence of any disruption in the glucose metabolism [247].

Global total and phosphorylated proteomic analyses in female fetal livers indicate that either HF/HS induced obesity or prenatal DHT exposure affect biological processes related to hepatic fatty acid metabolism. We found that the expression of total Vasp, and Masv was modulated by the interaction of diet and DHT in female fetal livers. Vasp is a protein that increase hepatic fatty acid oxidation in mice [248] and Masv, is a protein that has been shown to be dissociated from the mitochondria in livers with steatohepatitis, impairing the inflammatory response in front of dsRNA challenge and promoting necrosis [249]. Another protein that showed differential expression was Forkhead box protein O1 (FOXO1), phosphorylated in serine 284 ($FOXO^{S284}$). FOXO1 is the most abundant protein in the insulin sensitive tissue such as liver and pancreas [250]. In our study (paper IV), the phosphorylation of Foxo1 was increased in fetal livers from dams exposed to DHT and decreased in fetal livers from mothers fed HF/HS-diet. The same phosphorylation profile was shown for $Rptor^{S863}$, a protein that is part of the mechanistic target of rapamycin complex 1 (mTORC1) signaling that promotes hepatic lipogenesis by activating sterol regulatory element-binding transcription factor (SREBP) [251]. As neither the phosphorylation of $FOXO^{S284}$ [252] nor $Rptor^{S863}$ have been studied previously, one may speculate that these phosphoylations may have a biologically significant effect in our study.

In proteomic analysis of placenta and fetal liver, COMT was differentially phosphorylated in Serine 261 ($COMT^{S261}$) in female fetal liver and also in placenta from female fetus. In fetal liver, $COMT^{S261}$ showed an increased phosphorylation in livers from female fetuses exposed to HF/HS induced obesity, while in placenta, $COMT^{S261}$ was affected by the interaction of the two factors, diet and injection, with decreased phosphorylation in mice fed control diet and exposed to DHT and increased phosphorylation in fetal liver from obese mothers, regardless the injection.

COMT is an enzyme that degrades catechol compounds like catecholamines, like adrenaline, noradrenaline, and 3,4-dihydroxyphenylacetic acid (DOPAC, a dopamine metabolite), into less active compounds, such as metanephrine, normetanephrine and homovanillic acid (HVA), respectively [253, 254]. COMT also methylate catechol estrogens, converting them to methoxy estrogens such as 4- methoxyestradiol (4-MeO-E2) and 2-methoxyestradiol (2-MeO-E2) [255]. Also the enzyme metabolize catechol estrogens and some drugs such L-DOPA [255]. The enzyme has been studied mostly because its polymorphism that are related to anxiety [256, 257], schizophrenia [258] and other psychiatric disorders [259]. In pregnancy, dysregulation of COMT enzyme activity could play a role in steroidogenic metabolism because low plasmatic 2-methoxyestradiol and 2-methoxyestrone have been related to preeclampsia [260] although

also higher levels of 2-methoxyestradiol have been detected in those patients in absence of changes in COMT expression in placenta [261]. A previous study demonstrated phosphorylation in $COMT^{S260}$ in several tissues from rats by using electrospray ionization with ion-trap tandem mass spectrometry (ESI-IT-MS/MS) [262]. However, there is no previous report on the activity of the enzyme in humans or animals related to these phosphorylations, and no report whether the phosphorylation causes an activation or inhibition of the enzyme. Although we did not find other signs of alteration in the catecholamine metabolism in placenta, (neither the gene expression of enzymes that degrades catecholamine nor the mRNA of their transporters was altered), the level of noradrenaline in plasma from mothers injected with DHT was lower. Most interesting is the fact that in F1 female generation, the hepatic mRNA expression of *Comt* was affected by the interaction of both diet and DHT exposure, decreased in animals from dams exposed to DHT and fed with control diet, and increased in animals from mother exposed to DHT and fed with HF/HS diet, compared to the vehicle injected group. In mouse liver, COMT is widely expressed and its activity is the highest between all the peripheral tissues [254]. It had demonstrated to have lower activity and protein expression in liver from spontaneously hypertensive male rats [263].

6 CONCLUDING REMARKS

Besides of the pregnancy complications women with PCOS display, it seems that the syndrome by itself has its own molecular signatures that probably take the women with PCOS to a more sensitive and vulnerable state which might cause long-term health consequences to their offspring. Moreover, it seems that the sympathetic nervous system is involved in the development of observed alterations and may play and important role in the pathophysiology of the syndrome during pregnancy.

The main conclusions of the papers included in this thesis are:

Paper I

Pregnant women with PCOS without any adverse pregnancy outcome display abnormal steroidogenesis and altered placental expression of steroidogenic enzymes and molecules related to fetal growth as was determined by the activation of STAT-3.

Paper II

EA treatment in a hyperandrogenic state during pregnancy alters the expression of molecules related to angiogenesis process and sympathetic modulation that compromised fetal growth.

Paper III

Diet induced maternal obesity and androgen exposure during pregnancy induce hepatic triglyceride accumulation and dysregulation of *de novo* lipogenesis in mothers, whereas in fetal liver this effect is not seen. Importantly, maternal obesity and prenatal androgenization affect the expression of important regulators of hepatic glucose metabolism that could affect the liver function in the offspring at adult age.

Paper IV

The novel COMT phosphorylation in fetal liver and placenta in mice and its altered gene expression in the liver of female offspring demonstrate that the sympathetic nervous system could play a role in the metabolic, reproductive or behavioral disturbances known in the offspring of PCOS.

7 FUTURE PERSPECTIVES

There are scarce and redundant information about the effects of maternal androgen exposure on fetal development and developmental origin of the disease. It is known that women with PCOS affect the long-term health of their offspring, but the mechanisms are still poorly studied.

The more specific questions that arise at the end of this work are related to the effects of the molecular alterations, which need be addressed in my future work:

1. As we demonstrated activation of STAT-3 in placentas from women with PCOS: Is placental nutrient transport affected in these women? What phenotype is the most affected? What is the metabolic state of the children born to mother with PCOS that display these abnormalities in placenta?
2. What is the specific role of the the alteration of NGF in fetal growth? Is the alteration of NGF common with others model of intrauterine growth restriction?
3. Was the decreased expression of mRNA of *pparg* seen in fetal liver a consequence of a direct effect of maternal androgen exposure?
4. What is the effect of the novel phosphorylation of COMT in the catecholaminergic metabolism? Why was the phosphorylation of COMT increased in HF/HS-DHT group whereas it was decreased in CD-DHT? What is the diet factor that is able to increase the phosphorylation of the enzyme?
5. As it is known that offspring born to mother with PCOS display anxiety-like behaviour, does the novel phosphorylation have a role in that phenotype?

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